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OXIDACIÓN LIPÍDICA EN PRODUCTOS LÁCTEOS: INFLUENCIA DE LA ADICIÓN DE ÁCIDOS GRASOS FUNCIONALES

Tesis Doctoral

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Resumen Tesis de María del Carmen García Martínez

OXIDACIÓN LIPÍDICA EN PRODUCTOS LÁCTEOS: INFLUENCIA DE LA ADICIÓN DE ÁCIDOS GRASOS FUNCIONALES

La oxidación lipídica es la alteración más importante que ocurre durante el procesamiento y la conservación de los alimentos ya que la aparición de olores y sabores característicos del desarrollo de la rancidez disminuye la aceptabilidad de los alimentos (1). Por otra parte, la ingestión de compuestos de oxidación no volátiles puede tener efectos negativos sobre la calidad nutricional y seguridad de los alimentos (2, 3).

Aun cuando los productos lácteos contienen mayoritariamente lípidos saturados, con baja susceptibilidad a la oxidación, existen en el mercado numerosos tipos de productos lácteos funcionales en los que se sustituye total o parcialmente la grasa láctea por aceites de origen vegetal o marino, por sus propiedades beneficiosas para la salud, como los ácidos grasos poliinsaturados omega-3 o mediante el incremento de ácido linoleico conjugado. La revisión bibliográfica realizada muestra que aún existe escasa información sobre la oxidación lipídica en productos lácteos funcionales. En la mayoría de estudios, realizaron medidas indirectas de oxidación lipídica, que ofrecen información sobre una etapa concreta del proceso de autoxidación, como son el índice de peróxidos (IP), TBARS, pérdida de sustrato, análisis sensorial, análisis de volátiles, etc. Prácticamente no hay estudios en productos comercializados, y entre éstos la mayoría es sobre productos lácteos simulados (4, 5, 6).

Otros productos que resultan relevantes para su estudio son las fórmulas infantiles, que simulan la leche materna y en su composición se combinan agentes oxidantes como metales con sustratos muy susceptibles a la oxidación, como son los ácidos grasos poliinsaturados. Además en su preparación se utilizan procesos industriales con elevadas temperaturas para conseguir productos en polvo y con larga caducidad.

El objetivo general de esta tesis es abordar el estudio del proceso de oxidación en productos lácteos, con especial atención a los siguientes aspectos:

- Determinación de los compuestos de oxidación presentes en una gran variedad de productos lácteos comercializados, tradicionales y funcionales: evaluación de métodos oficiales y desarrollo de métodos alternativos.

-Estudios de oxidación específicos en fórmulas infantiles modelo y comercializadas, para determinar el efecto de tratamientos térmicos de esterilización, la adición de ácidos grasos poliinsaturados y las condiciones de almacenamiento en la formación de compuestos oxidados.

- Estudios de oxidación específicos en sistemas modelo y productos comercializados con ácido linoleico conjugado (CLA), para identificar y cuantificar los compuestos de oxidación formados, volátiles y no volátiles, y evaluar la influencia de la temperatura y acción de antioxidantes fenólicos, entre otras variables, en la cinética de oxidación.

Determinación de los compuestos de oxidación en productos lácteos comercializados, tradicionales y funcionales.

Se ha puesto a punto una metodología basada en una combinación de técnicas cromatográficas de adsorción y exclusión para la evaluación de la oxidación lipídica en productos lácteos y aplicado a muestras de leche esterilizada y UHT, leche en polvo, leche concentrada, yogures y fórmulas infantiles; así como a preparados lácteos funcionales con ácidos grasos poliinsaturados omega-3, CLA y estanoles vegetales. Las muestras fueron evaluadas en el tiempo de adquisición y al final de su vida útil.

Los resultados demuestran que sólo los productos lácteos funcionales que han sido enriquecidos con ácidos grasos poliinsaturados omega-3 ó ácido linoleico conjugado (CLA) son susceptibles a oxidación, presentando niveles significativamente más altos de triglicéridos oxidados o poliméricos incluso antes de su fecha de caducidad.

En el caso de los productos con omega-3 el aumento de los niveles de oxidación se debe al incremento de triglicéridos monómeros oxidados mientras que en las muestras con CLA los compuestos poliméricos son mayoritarios. La formación de polímeros de elevado peso molecular en las muestras con CLA y los sorprendentemente bajos niveles de índice de peróxidos encontrados indican que la cinética de oxidación del aceite rico en CLA es diferente a la ampliamente aceptada para sustratos no conjugados y justifica la realización de estudios complementarios para definir medidas de oxidación adecuadas para el control de calidad de estos productos (7).

Estudios de oxidación en fórmulas infantiles modelo y comercializadas.

Se han realizado estudios de oxidación específicos en productos lácteos muy susceptibles a la oxidación lipídica, las fórmulas infantiles, y de extraordinaria importancia porque suponen la única fuente de nutrientes de los lactantes y es esencial garantizar su calidad y seguridad. Las fórmulas infantiles constituyen un excelente modelo para determinar si el proceso de elaboración y el tratamiento de esterilización influyen en la oxidación lipídica ya que son particularmente susceptibles por su alto contenido en ácidos grasos poliinsaturados y minerales con acción prooxidante.

Se elaboraron fórmulas infantiles modelo que simularon fórmulas de inicio y continuación con una mezcla de aceites con la composición de ácidos grasos normalizada por la regulación española que recoge las especificaciones de la normativa europea (Real Decreto, 2008), y con fines comparativos dos fórmulas con uno de los aceites utilizados en la formulación, esto es, un aceite resistente a la oxidación (girasol alto oleico) y un aceite muy susceptible a la oxidación (pescado), con o sin hierro en la formulación. La relación caseinato sódico:proteínas del suero de la leche fue 1:4 and 4:1 para las fórmulas de inicio y las de continuación, respectivamente.

Los resultados mostraron que el tratamiento de esterilización no produjo un aumento significativo en los compuestos de oxidación en fórmulas de inicio y continuación elaboradas con composición similar a las comercializadas aunque se observaron disminuciones significativas de tocoferoles en las fórmulas de inicio con la consecuente pérdida de protección frente a la posibles deterioros oxidativos durante el posterior periodo de comercialización. El principal agente protector fue la fracción proteica, especialmente el caseinato sódico, presente en mayor concentración en las fórmulas de continuación (8, 9).

En el estudio de las fórmulas infantiles en polvo comercializadas se evaluó la influencia de las condiciones de conservación en la estabilidad oxidativa y se introdujo por primera vez la determinación de dos fracciones diferenciadas de lípidos en fórmulas infantiles: la fracción superficial, obtenida mediante extracción con hexano; y la fracción encapsulada, obtenida tras disgregar la matriz de proteínas e hidratos de carbono en medio básico fuerte (con solución etanólica de amoníaco) y posterior extracción con éter dietílico y pentano. Las muestras fueron almacenadas a 25, 30 y 37ºC durante 3 meses. La evaluación de los lípidos totales extraídos no mostró diferencias en estabilidad oxidativa mientras que el análisis específico de la fracción superficial, que aunque minoritaria (7,5% de los lípidos totales) es más susceptible a la oxidación, mostró un aumento significativo de compuestos de oxidación a todas las temperaturas, y fue consistente con la aparición de rancidez en algunas muestras. La pérdida de tocoferoles fue consistente con el aumento de los niveles de oxidación observados. Estos resultados demuestran la necesidad de analizar separadamente la fracción lipídica superficial en fórmulas infantiles en polvo para determinar su estado de oxidación (10).

Estudios de oxidación en el aceite rico en CLA adicionado a productos lácteos funcionales.

Se ha utilizado el aceite Tonalin TG80, suministrado por la empresa Cognis, ya que es el adicionado como aceite rico en CLA en los productos lácteos evaluados. El aceite Tonalin es obtenido mediante isomerización alcalina del aceite de cártamo, también utilizado en estos estudios con fines comparativos. El contenido de ácido linoleico (C18:2 9*c*, 12*c*) (74,7%) en el aceite de cártamo es similar al del contenido total de CLA (76,8%) (38,2% de C18:2 9*c*, 11*t* y 38,6% de C18:2 10*t*, 12*c*). Los ensayos de oxidación se han realizado a 100ºC en Rancimat, a 60ºC y 40ºC en estufa y a temperatura ambiente (25ºC). La evolución de la formación de compuestos primarios y secundarios de oxidación ha mostrado enormes diferencias entre el aceite Tonalin y su precursor aceite de cártamo, independientemente de las condiciones del ensayo.

El índice de peróxidos del aceite de cártamo mostró un aumento progresivo desde el principio y cuando se agotaron los tocoferoles, alcanzó un valor de 152 meq O_2/kg, mientras que en el aceite Tonalin el IP apenas aumentó durante el periodo de inducción, sin embargo la

concentración de polímeros fue incrementándose desde el principio y alcanzaron niveles en torno al 15% en el punto de agotamiento de los tocoferoles (11).

Los resultados obtenidos demuestran que existen mecanismos de oxidación diferentes entre los aceites ricos en ácido linoleico, que se oxidan según la teoría ampliamente aceptada a través de la formación de hidroperóxidos, y los aceites ricos en ácido linoleico conjugado, en los cuales los compuestos primarios de oxidación no son hidroperóxidos sino polímeros con enlaces peróxido de elevado peso molecular. El perfil de volátiles es también muy diferente en el aceite rico en CLA, pues no predomina exclusivamente la formación de hexanal sino también heptanal y otros compuestos procedentes de rutas alternativas a la de formación de hidroperóxidos. Estos resultados demuestran la invalidez del índice de peróxidos como medida de control de oxidación de aceites ricos en CLA, la única que consta en las especificaciones de los productos comercializados (11, 12).

Estrategias para prevenir la oxidación de aceites ricos en CLA: adición de antioxidantes y microencapsulación.

Con objeto de proteger los aceites ricos en CLA de la oxidación se han utilizado dos estrategias, la adición de diferentes tipos de tocoferoles y sinergistas, y la técnica de microencapsulación. Los antioxidantes seleccionados fueron el alfa, delta y gamma-tocoferol, así como sus combinaciones con la mezcla sinérgica APL (palmitato de ascorbilo + lecitina), que se adicionaron a los aceites una vez desprovistos de sus antioxidantes, mediante cromatografía en columna de alúmina.

Los resultados demuestran que el delta-tocoferol, muy efectivo en aceites convencionales, lo es mucho menos en el aceite Tonalin. Asimismo, la adición de APL es en general menos protectora en el aceite Tonalin. Estas mezclas son utilizadas normalmente en productos comerciales por tanto es necesario evaluar la eficacia de antioxidantes alternativos (13).

Los ensayos con aceite de Tonalin microencapsulado (TM), suministrados por una empresa, se realizaron aplicando la separación diferencial de fracción libre y encapsulada. Se evaluaron las principales características físico-químicas, como la temperatura de transición vítrea y el tamaño y la distribución medios de los glóbulos de aceite. El nivel de oxidación se evaluó mediante la determinación de la pérdida de sustrato, determinación de polímeros (14) y el contenido en tocoferoles.

Los resultados mostraron que el aceite libre se oxidó a mayor velocidad que el aceite encapsulado. Los resultados demuestran que la microencapsulación es muy útil para proteger aceites ricos en CLA utilizados como ingredientes funcionales en productos lácteos (15).

En resumen, la contribución general de esta Tesis ha sido aumentar el conocimiento de los mecanismos de oxidación lipídica en productos lácteos, concretamente en los altamente susceptibles a esta alteración, es decir, los productos lácteos funcionales enriquecidos en ácidos grasos poliinsaturados omega-3 y ácido linoleico conjugado. Así mismo, se han propuesto nuevos métodos para evaluar el estado de oxidación de estos productos, cuyo control es esencial para garantizar que los lípidos bioactivos adicionados proporcionen los beneficios nutricionales esperados.

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Memoria que presenta María del Carmen García Martínez
para optar al grado de Doctor en Ciencias de la Alimentación
por la Universidad Autónoma de Madrid.

Trabajo realizado bajo la dirección de:
Dra. Gloria Márquez Ruiz
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Instituto de Ciencia y Tecnología de Alimentos y Nutrición (CSIC)

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CERTIFICAN:

Que el trabajo titulado: "OXIDACIÓN LIPÍDICA EN PRODUCTOS LÁCTEOS FUNCIONALES: INFLUENCIA DE LA ADICIÓN DE ÁCIDOS GRASOS POLIINSATURADOS", que constituye la Memoria de la que es autora María del Carmen García Martínez, ha sido realizado en el Instituto de Ciencia y Tecnología de Alimentos y Nutrición del C.S.I.C. bajo su dirección y que cumple las condiciones exigidas para optar al grado de Doctor por la Universidad Autónoma de Madrid y, por tanto, autorizan su presentación.

Y para que conste a los efectos oportunos, firmamos el presente Certificado en Madrid a 10 de septiembre de 2018.

Fdo. Gloria Márquez Ruiz Fdo. Francisco Javier Fontecha Alonso

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A mis padres

A Jorge

A nuestro hijo Roberto

Al hij@ que estoy deseando conocer pronto

Abreviaturas.

A: Densidad aireada

a* y b* : Coordenadas de cromaticidad

AG: Ácidos grasos libres

AGPI: Ácidos grasos poliinsaturados

APL: Palmitato de ascorbilo mas lecitina

ASE: Área de superficie específica

aw: Actividad de agua

BHA: Hidroxibutilanisol

BHT: Butilhidroxitolueno

C.P.: Compuestos polares totales

CG: Cromatografía de gases

CLA: Ácido linoleico conjugado

$d_{v,0,5}$: Diámetro promedio de gotas de aceite

$d_{(v,0,9)} - d_{(v,0,1)}$: Rango de diámetros de gotas

D [3,2]: Diámetro de Sauter

DG: Diglicéridos

DHA: Ácido docosahexaenoico

DSC: Calorimetría diferencial de barrido (Differential scanning calorimetry)

EE: Eficacia de encapsulación

ENC: Fracción de aceite encapsulado

EPA: Ácido eicosapentaenoico

ESR: Espectroscopía de espín electrónico (Electron spin resonance spectroscopy)

FTIR: Espectroscopia Infrarroja de Transformada de Fourier

HPLC: Cromatografía líquida de alta resolución (High-performance liquid chromatography)

HPSEC: Cromatografía de exclusión molecular de alta resolución (High-performance size-exclusion chromatography)

HR: Humedad relativa

IP: Índice de peróxidos

LA: Ácido linoleico

LIB: Fracción de aceite libre

OSI: Índice de estabilidad oxidativa (Oxidative stability index)

P: Densidad empacada

p: Probabilidad

PI: Periodo de inducción

POL: Polímeros

RMN: Resonancia magnética nuclear

SPME: Microextracción en fase sólida

TBARS: Método de medición de sustancias reactivas al ácido tiobarbitúrico (Thiobarbituric acid reactive substances)

TBHQ: Terbutil Hidroquinona

Tg: Temperatura de transición vítrea

TGD: Dímeros de triglicéridos

TGMox: Triglicéridos monómeros oxidados

TGP: Polímeros de triglicéridos

TM: Aceite Tonalin® microencapsulado

TOC: Tocoferoles

UHT: Ultrapasteurización (Ultra High Temperature)

Índice.

1.- Introducción.

Los productos lácteos funcionales han experimentado un gran desarrollo en los últimos años, en gran parte debido a la facilidad de incorporación de ingredientes funcionales a matrices lácteas y a que son productos de consumo diario para la mayoría de la población (Sloan, 2016; Nachai 2015; Gulseven y col., 2014; Huth y col., 2006). Los productos lácteos funcionales son de dos tipos (Kanekaian, 2014). Por una parte, productos lácteos enriquecidos en sus componentes naturales como el calcio y las vitaminas A, D y E, o en otros ausentes de forma natural como la fibra, microorganismos prebióticos o compuestos probióticos, que mejoran el estado inmunológico, jalea real para aminorar estados de cansancio y fatiga, isoflavonas de la soja para reducir los síntomas de la menopausia, péptidos con actividad antihipertensiva y esteroles vegetales para reducir los niveles de colesterol. Por otra parte, hay productos lácteos en los que se sustituyen componentes con efectos potencialmente negativos por otros que puedan resultar beneficiosos. Es el caso de los derivados lácteos en los que parte o la totalidad de la grasa de leche se ha sustituido por aceites vegetales o mezcla de éstas con aceites de pescado o algas. De esta forma se consigue reducir el contenido en grasa saturada e incrementar el contenido en ácidos grasos poliinsaturados con efectos positivos en la salud, como los ácidos grasos poliinsaturados omega-3 (AGPI w-3) (Shahidi, 2015) o el ácido linoleico conjugado (CLA) (Yang y col., 2015). En contraste con la baja susceptibilidad a la oxidación de la leche y los productos lácteos convencionales, que contienen mayoritariamente lípidos saturados, los productos lácteos funcionales con ácidos grasos poliinsaturados son sustratos fácilmente oxidables. Los efectos que los nuevos procesos de elaboración y las condiciones de almacenamiento puedan tener en la formación de compuestos oxidados no deseados son por tanto de gran relevancia para garantizar su calidad y seguridad y, sin embargo, han sido hasta ahora escasamente estudiados (García-Martínez y Márquez-Ruiz, 2009; Rodríguez-Alcalá y Fontecha, 2007; Rodríguez-Alcalá y col., 2014).

1.1.-Oxidación lipídica.

La oxidación lipídica es una de las alteraciones más importantes que ocurren durante el procesamiento y la conservación de los alimentos ya que la aparición de olores y sabores característicos del desarrollo de la rancidez disminuye la aceptabilidad de los alimentos (Frankel, 2005) y la ingestión de compuestos de oxidación no volátiles puede tener efectos negativos sobre la calidad nutricional y seguridad de los alimentos (Dobarganes y Márquez-Ruiz, 2003; Kanner, 2007).

En este apartado 1.1. se resumen las características generales de la oxidación lipídica, incluyendo una breve descripción del proceso de autoxidación -el mecanismo principal de oxidación en los lípidos de alimentos-, de los compuestos de oxidación formados, de las variables de mayor relevancia que intervienen en el proceso y de los métodos más utilizados para la evaluación de la oxidación, con especial énfasis en los aplicados en estudios de productos lácteos.

1.1.1.- Proceso de autoxidación y compuestos formados.

La autoxidación es el mecanismo principal de oxidación en los lípidos de alimentos y es un proceso autocatalítico de reacciones en cadena, que transcurre a través de la formación de radicales libres y consta de cuatro fases: iniciación, propagación, ramificación y terminación. Los mecanismos y cinética del proceso de autoxidación han sido extensamente revisados durante décadas por los investigadores más relevantes en esta área (Grosch, 1987; Porter y col., 1995; Brimberg y Kamal-Eldin 2003; Frankel, 2005; Choe y Min, 2007; Schneider 2009, Hammond y White, 2011; Barden y Decker, 2016). Los triglicéridos constituyen más del 95% de los lípidos en los alimentos y, por tanto, la oxidación lipídica ocurre fundamentalmente en las cadenas insaturadas de sus restos acilo.

En la fase de iniciación (1) se forma un radical alquílico por la abstracción de un hidrógeno H· de un grupo metileno adyacente al doble enlace de una molécula insaturada RH, por exposición a energía luminosa, calorífica o catálisis metálica.

Es la reacción menos conocida de todo el proceso. La presencia de hidroperóxidos y metales en cantidades traza parece tener un papel importante en la generación de radicales que actúan como iniciadores.

En la etapa de propagación el radical formado R· reacciona con el oxígeno atmosférico para formar un radical peroxilo ROO· y éste reacciona con una nueva molécula insaturada para originar el hidroperóxido ROOH (compuesto primario de oxidación) y un nuevo radical alquílico R· que repite la secuencia de reacciones con otra molécula insaturada. Los radicales alílicos se forman un orden de magnitud más lentamente que los bialílicos por la mayor estabilización por resonancia del electrón desapareado. La reacción limitante de esta fase es la (3) que permite la acumulación de hidroperóxidos y depende de la fuerza del enlace C-H, siendo más lábil en la posición alílica.

La ramificación consiste en la descomposición de hidroperóxidos incrementando la concentración de radicales libres. La reacción de descomposición puede ser monomolecular (4) y bimolecular (5) aunque la segunda está más favorecida porque requiere menor energía de activación.

La terminación es la eliminación de radicales del sistema (peroxilo, alcoxilo, acilo, hidroxilo e hidrógeno) para formar compuestos no radicalarios estables. La interacción de dos radicales peroxilo (6) es la más importante de las reacciones de terminación a baja temperatura. Después de la formación de los primeros radicales, las tres etapas de la oxidación ocurren simultáneamente, pero a diferentes velocidades que cambian a lo largo del proceso.

Iniciación:	$RH \rightarrow R^{\bullet} + H^{\bullet}$		(1)
Propagación:	$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$		(2)
	$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$		(3)
Ramificación:	$ROOH \rightarrow RO^{\bullet} + HO^{\bullet}$	Monomolecular	(4)
	$2\ ROOH \rightarrow ROO^{\bullet} + RO^{\bullet} + H_2O$	Bimolecular	(5)
Terminación:	$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2$		(6)
	$R^{\bullet} + R^{\bullet} \rightarrow RR$		(7)
	$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR$		(8)
	$R^{\bullet} + RO^{\bullet} \rightarrow ROR$		(9)
	$R^{\bullet} + H^{\bullet} \rightarrow RH$		(10)
	$RO^{\bullet} + H^{\bullet} \rightarrow ROH$		(11)
	$ROO^{\bullet} + H^{\bullet} \rightarrow ROOH$		(12)
	$R^{\bullet} + HO^{\bullet} \rightarrow ROH$		(13)

1.1.1.1.- Compuestos primarios de oxidación.

Se forman tanto por reacciones de propagación como de terminación. Son compuestos inestables, que se descomponen fácilmente en radicales (principalmente alcoxil y hidroxil radicales) para formar productos secundarios de oxidación. En condiciones de temperaturas bajas y moderadas constituyen una fracción muy importante de los compuestos de oxidación. Los hidroperóxidos se forman y se descomponen simultáneamente, pero durante la primera etapa del proceso de oxidación y hasta el final del periodo de inducción la velocidad de formación es mucho mayor y es donde se observa el aumento de los hidroperóxidos. Cuando se acumulan altos niveles de hidroperóxidos su descomposición se produce más rápidamente que su formación, y aumenta la velocidad de oxidación exponencialmente.

Las estructuras y distribución de los principales hidroperóxidos se han resuelto en sistemas modelo de oleato, linoleato y linolenato de metilo (Frankel, 2005). Sin embargo, la formación de hidroperóxidos a partir del ácido linoleico conjugado (CLA) aún no está clara y genera controversia. Únicamente en

presencia de cantidades demasiado elevadas y no realistas (20% en peso) de tocoferol (donador de hidrógeno) ha sido identificada una mezcla compleja de hidroperóxidos conjugados en metil *cis*-9, *trans*-11-octadecanoato oxidado (Hämäläinen y col., 2002). La autoxidación del CLA parece que ocurre a través de reacciones de condensación rápidas que producen productos de polimerización desde los primeros estados de la oxidación lipídica (Luna *y col.*, 2007).

1.1.1.2.- Compuestos secundarios de oxidación.

Se forman a partir de los hidroperóxidos a través de distintos tipos de reacciones, y pueden agruparse en tres clases, de acuerdo con el rango de peso molecular de los compuestos obtenidos: *monómeros oxidados*, compuestos de peso molecular similar al de los triglicéridos no oxidados; *volátiles*, compuestos de menor peso molecular que los triglicéridos no oxidados; y *compuestos diméricos y poliméricos*, de peso molecular muy superior al de los triglicéridos no oxidados.

Monómeros oxidados.

La vía principal de descomposición de los hidroperóxidos es la escisión homolítica del grupo hidroperóxido formando radicales hidroxilo y alcoxilo (Min y Boff, 2002). El radical alcoxilo puede participar en diferentes reacciones que conducen a la formación de compuestos con funciones epoxi, hidroxi y ceto, entre otras, que constituyen, junto a los hidroperóxidos, el grupo mayoritario originado por degradación oxidativa a temperatura ambiente o moderada (Frankel, 2005).

Componentes volátiles.

En los radicales alcoxilo puede también tener lugar la escisión homolítica del enlace C-C a ambos lados del citado radical (posición β respecto al O), dando lugar a la formación de volátiles. Se produce un aldehído estable y un radical que puede reaccionar con otros radicales en juego dando lugar a compuestos estables o reaccionar con el oxígeno reiniciándose así de nuevo la cadena de propagación (Kochhar, 1993). Reacciones similares ocurren tras la escisión al

otro lado de la cadena (que porta el grupo carboxilo), que darían lugar a ácidos, hidroxiácidos o aldehídos ácidos unidos al glicerol en el caso de los triglicéridos formando parte así de una molécula no volátil.

La compleja mezcla de compuestos volátiles formados sólo suponen una pequeña parte del total de los productos de oxidación pero poseen una extraordinaria importancia desde el punto de vista sensorial (Frankel, 2005; Grosch, 1987).

Dímeros y polímeros.

Los radicales alcoxilo pueden participar así mismo en reacciones de condensación con otros radicales para formar dímeros y polímeros, fundamentalmente de triglicéridos, con enlaces entre monómeros de triglicéridos de tipo puente C-C, puente éter (C-O-C) y puente peróxido (C-O-O-C) (Dobarganes y Márquez-Ruiz, 2000). Aunque los dímeros y polímeros son los productos mayoritarios a elevada temperatura, a la que predominan las uniones tipo éter y C-C (Neff y col., 1988; Dobarganes y Márquez-Ruiz, 2007; Sánchez-Muniz y col., 2007), también se forman a temperaturas bajas al final del periodo de inducción (Márquez-Ruiz y col., 2000; Márquez-Ruiz y Dobarganes 2006).

La Figura 1. recoge de forma esquemática estructuras representativas de los distintos compuestos de oxidación formados a partir de los triacilgliceroles, donde se observan las principales funciones oxigenadas presentes y enlaces entre monómeros de triglicéridos.

Además de la autoxidación, existen otros mecanismos de oxidación como la fotooxidación y la oxidación enzimática. La fotooxidación ocurre exclusivamente en presencia de luz y por mediación de compuestos fotosensibilizadores, como los pigmentos naturales de carnes (hemoglobina, mioglobina) y verduras (clorofila y feofitina), y se diferencia en que las reacciones de formación de hidroperóxidos transcurren mediante adición directa de oxígeno al doble enlace. La oxidación enzimática, catalizada fundamentalmente por el enzima lipoxigenasa en alimentos vegetales frescos, da lugar a los mismos hidroperóxidos que la autoxidación, diferenciándose en la estereoquímica y en

las proporciones relativas debido a que la reacción es estereoespecífica y regioselectiva.

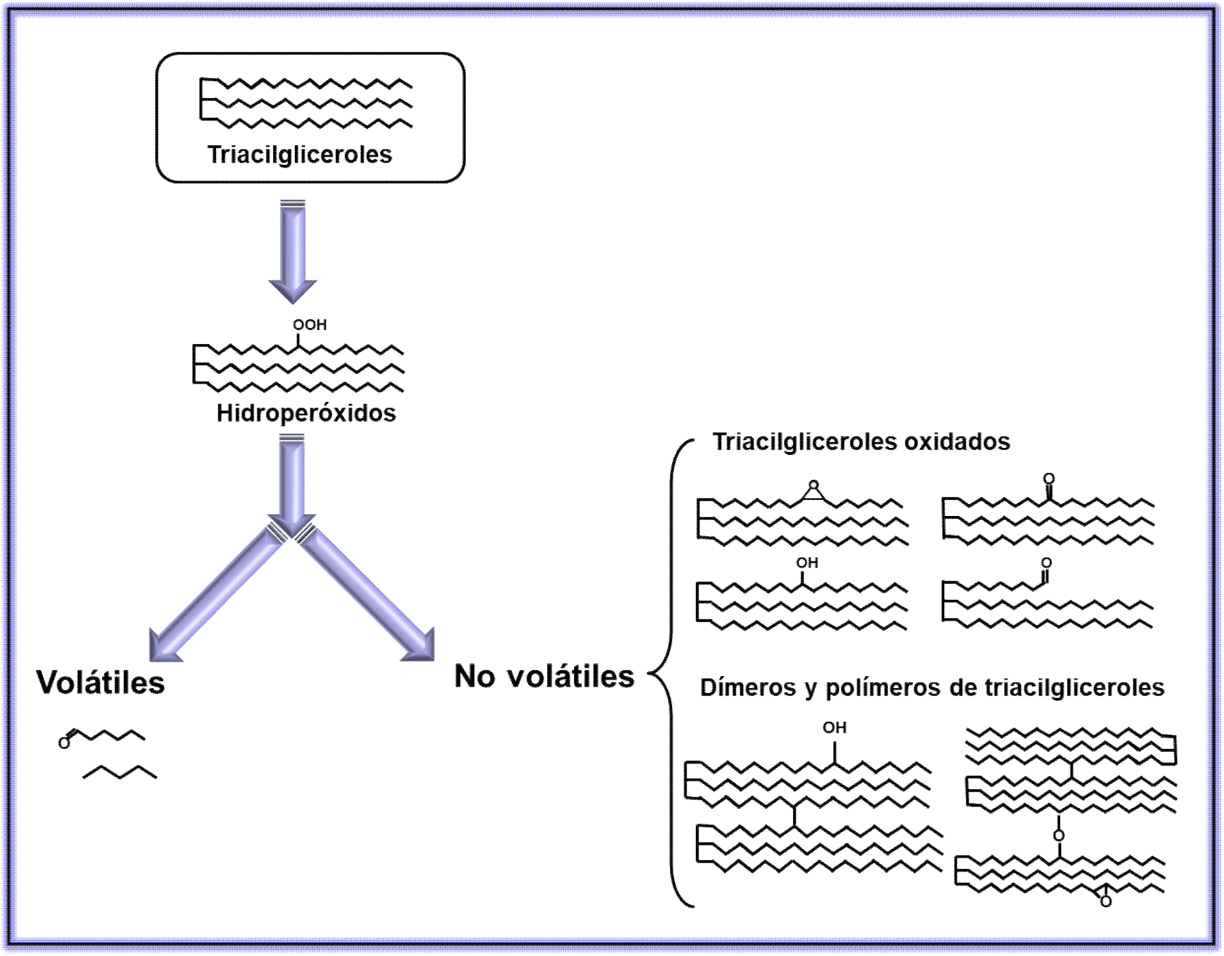

Figura 1.- Esquema representativo de las estructuras formadas, volátiles y no volátiles como resultado de la autoxidación de los triacilgliceroles.

1.1.2.- Factores generales que influyen en la oxidación.

Numerosas variables pueden ejercer una influencia importante sobre el desarrollo oxidativo, entre las cuales destacan la temperatura, disponibilidad de oxígeno, presencia de antioxidantes y prooxidantes, y grado de insaturación (Frankel, 2005). La complejidad aumenta enormemente en sistemas dispersos, como las emulsiones y alimentos deshidratados, fundamentalmente debido a la heterogeneidad de la distribución de los lípidos (Márquez-Ruiz y col., 2013). Los productos lácteos engloban una gran variedad de sistemas dispersos, como por ejemplo la leche (emulsión grasa en agua), la leche en polvo (emulsión

deshidratada) y las fórmulas infantiles (grasas microencapsuladas), donde los glóbulos de grasa están dispersos en una matriz de hidratos de carbono y proteínas y es necesario distinguir entre distintas fracciones (Vignolles y col., 2007). En la mayoría de los casos es difícil evaluar el efecto de un factor específico en la oxidación global porque muchos actúan simultáneamente o están interrelacionados. En general los factores se pueden dividir en factores intrínsecos, es decir, inherentes a la composición de los lípidos (grado de insaturación, contenido de antioxidantes y prooxidantes), y los factores extrínsecos (oxígeno, luz, temperatura).

1.1.2.1. Grado de instauración.

Se ha comprobado que la velocidad de autoxidación para los 3 principales ácidos grasos constituyentes de los lípidos (oleico, linoleico y linolénico) es proporcional a 1:40-50:100 en lo que se refiere a la absorción de oxígeno y a 1:12:25 en cuanto a la formación de hidroperóxidos (Frankel, 2005). Los resultados indican que la oxidación se incrementa enormemente cuando se pasa del ácido oleico al linoleico y ello se debe a que, dada la posición de los dobles enlaces en el caso de los ácidos poliinsaturados, existe un sistema pentadiénico con un carbono en posición especialmente activada por la influencia simultánea de dos dobles enlaces. Cuando los ácidos se encuentran en mezclas, la velocidad de oxidación del más insaturado es determinante (Neff y col., 1994a; Frankel, 1998a).

1.1.2.2. Antioxidantes.

La adición de antioxidantes a los alimentos es la manera más efectiva y económica de retardar la oxidación. Las principales reacciones que justifican su acción implican la interacción con los radicales alquilo, alcoxilo y peroxilo, y la estabilización posterior de los radicales de moléculas antioxidantes entre ellas o con otros radicales del sistema, para formar productos estables. Actualmente existe una creciente tendencia al uso de antioxidantes naturales de ahí que los estudios sobre su acción y aplicación sean muy numerosos, y se han incluido en revisiones muy completas (Frankel, 2005, Frankel y col., 2008; Kamal-Eldin y

Appelqvist, 1996; Yanishlieva y Marinova, 2003; Aruoma y Cuppett, 1997; Shahidi, 2000; Kiokias y col., 2008).

Los antioxidantes primarios (AH) interrumpen la reacción en cadena de radicales libres mediante la donación de hidrógeno o electrones, convirtiéndolos en productos estables, tal y como se esquematiza a continuación:

$ROO^{\bullet} + AH \Leftrightarrow ROOH + A^{\bullet}$ (I/-I)

$R^{\bullet} + AH \rightarrow RH + A^{\bullet}$ (II)

$A^{\bullet} + ROO^{\bullet} \rightarrow ROOA$ (III)

$A^{\bullet} + A^{\bullet} \rightarrow AA$ (IV)

Aunque algunos antioxidantes primarios no son fenoles (etoxiquinina y anoxómero) la mayoría y los más efectivos son los de estructura fenólica con sustituyentes alquilo, naturales como los tocoferoles, flavonoides y catequinas, y sintéticos como el BHA, BHT, TBHQ (*terc*-butilhidroquinona) y galato de propilo. Estos antioxidantes producen radicales $A^{\bullet}$ estables mediante las reacciones I y II, poco reactivos en términos de propagación de la reacción, y compiten con el sustrato lipídico RH, presente en mucha mayor concentración, por los radicales peroxilo $ROO^{\bullet}$. En presencia del antioxidante, la velocidad de formación de hidroperóxidos está relacionada con el cociente de concentraciones de lípido y antioxidante

$d[ROOH] / dt = k_p R_i [LH] / (k_I [AH])$

donde k_p es la constante de velocidad de la reacción de propagación (3), R_i la velocidad de la reacción de iniciación (1) y k_I la constante de velocidad de la reacción (I). A temperaturas elevadas, la concentración de oxígeno es limitante y la reacción de inhibición (II) es más importante, así la velocidad de descomposición de los hidroperóxidos está entonces relacionada con el cociente de la concentración de oxígeno y antioxidantes:

$d[ROOH] / dt = k_p R_i [O_2] / (k_{II} [AH])$

donde k_{II} es la constante de velocidad de la reacción de inhibición (II).

Los radicales $A^{\bullet}$ se estabilizan por deslocalización electrónica en la estructura fenólica, así pues la efectividad del antioxidante está directamente relacionada con la estabilización por resonancia. En determinadas circunstancias, los antioxidantes fenólicos son menos efectivos por su tendencia a oxidarse o por reaccionar el radical resultante con el sustrato lipídico para iniciar la cadena autoxidativa.

Los tocoferoles son los antioxidantes primarios más abundantes en aceites y alimentos, los más utilizados para aumentar la estabilidad de los alimentos procesados y, en consecuencia los más estudiados (Kayden y Traber, 1993; Burton e Ingold, 1986; Niki y col., 1986; Kamal-Eldin y Appelqvist, 1996). Consisten en un anillo de cromano y una cadena de fitol lateral. Los isómeros α, β, γ y δ difieren en el número y posición de los grupos metilo en la parte fenólica del anillo de cromano. Todos poseen actividad vitamínica y el más potente *in vivo* es el R, R, R-α-tocoferol.

Los antioxidantes secundarios son compuestos que retardan la oxidación por otros mecanismos y pueden actuar como sinergistas de los antioxidantes primarios. Entre ellos, los agentes quelantes son principalmente compuestos que inactivan los iones metálicos, mediante formación de complejos de coordinación o modificaciones del potencial redox. Los compuestos quelantes más comunes son el ácido cítrico, el ácido fosfórico, el ácido etilendiaminotetraacético (EDTA), el ácido fítico y los fosfolípidos. Por otro lado, los secuestradores de oxígeno más utilizados son el ácido ascórbico y palmitato de ascorbilo. Probablemente las combinaciones con efecto sinérgico más conocidas son aquéllas entre tocoferoles y ácido cítrico o ácido ascórbico. La combinación tocoferol-ácido ascórbico-lecitina es particularmente efectiva para aceites poliinsaturados (Han y col., 1990, 1991).

1.1.2.3. Prooxidantes.

Entre los compuestos que aceleran la oxidación, destacan los metales de transición, como el cobre y el hierro. Se encuentran siempre presentes como trazas de impurezas, incluso son añadidos a ciertos alimentos como

micronutrientes. Su acción en la formación de radicales puede explicarse principalmente por la aceleración de la descomposición de hidroperóxidos mediante reacciones redox, a través de la formación de complejos de transición (Schaich, 1992).

1.1.2.4. Oxígeno.

La concentración de oxígeno en el medio influye significativamente en la cinética de oxidación (Cosgrove *y col.*, 1987; Brimberg, 1993). A presiones de oxígeno superiores a 100 mmHg, la velocidad de oxidación es independiente del contenido de oxígeno. Por el contrario, cuando el oxígeno está limitado y la presión parcial (PO_2) es inferior a 100 mmHg, la velocidad de formación de hidroperóxidos es dependiente de la presión de oxígeno de acuerdo con la ecuación:

$d[ROOH] / dt = A [PO_2 / (PO_2 + B)]$

Donde A y B son constantes asumiendo una velocidad constante de iniciación y cantidad de substrato.

1.1.2.5. Luz.

El efecto directo de la luz en los lípidos consiste en acelerar la autoxidación favoreciendo la formación de los radicales libres en la etapa de iniciación, por ejemplo actuando como catalizador de la abstracción del hidrógeno. Las radiaciones ultravioleta, junto a las visibles hasta 540 nm, son las que aceleran más la oxidación.

1.1.2.6. Temperatura.

En general, al aumentar la temperatura, se aceleran todas las reacciones de la cadena autoxidativa, por lo que la cantidad de compuestos obtenidos es mucho mayor a temperaturas elevadas (Dobarganes, 1998). Existe además una importante relación entre la temperatura y el oxígeno, porque la solubilidad del

oxígeno disminuye al aumentar la temperatura. La temperatura tiene un importante efecto en la reacción de propagación (3), pues ésta tiene una energía de activación superior a la de la reacción de iniciación y a la de la primera reacción de propagación del radical alquilo con oxígeno (2), aumentando la velocidad de (3) aproximadamente el doble cada 20°C entre 40°C y 100°C. Durante la fase de ramificación, a baja temperatura la velocidad de formación de hidroperóxidos es mayor que su descomposición, que tiene lugar a través de la vía monomolecular y por tanto los compuestos mayoritarios son triglicéridos monómeros oxidados. Sin embargo, a elevada temperatura, la velocidad de ramificación a través de la descomposición bimolecular es mayor que su formación, y los principales compuestos originados son dímeros y polímeros ya que los radicales con posibilidad de interaccionar son glicerídicos (Chang *y col.*, 1978; Dobarganes y Márquez-Ruiz, 1996; Dobarganes, 1998). La temperatura afecta las reacciones de terminación por influencia en la disponibilidad de oxígeno (Labuza, 1971; Karel, 1992). Así, los dímeros con enlaces peróxidos son más frecuentes en oxidación a temperaturas bajas por la abundancia relativa de radicales peroxilo y alquilo, frente a los puente éter y puente C-C, encontrados mayoritariamente a temperaturas elevadas por la predominancia de radicales alquilo y alcoxilo (Neff *y col.*, 1988).

1.1.2.7. Factores adicionales en sistemas heterofásicos.

Además de las variables que influyen en el desarrollo de la oxidación descritas anteriormente, existen factores adicionales en sistemas heterofásicos, que también tienen incidencia en el desarrollo de la oxidación lipídica (Velasco y col., 2002; Waraho y col., 2011; Berton-Carabin y col., 2014; Márquez-Ruiz y col., 2014). Las emulsiones y productos en polvo constituyen sistemas lipídicos heterofásicos, e incluyen respectivamente a la leche y una gran variedad de productos lácteos (yogures, batidos, nata, leche fermentada, etc.), y a la leche en polvo y fórmulas infantiles.

Emulsiones.

En estos sistemas, la interfase o región interfacial que rodea las gotículas (mezcla de lípidos, agua y emulsionante con un grosor típicamente de unos pocos nanómetros) puede actuar como una barrera a la penetración o difusión de especies moleculares y sus modificaciones (cambios en la carga eléctrica, concentración, permeabilidad, etc.) pueden influir enormemente en el desarrollo de la oxidación (Berton y col., 2011). De ahí el cambio de conformación de ciertas proteínas que conduce a la exposición de sus cadenas peptídicas en la interfase influye en la oxidación lipídica. Un ejemplo de ello es el efecto inhibidor de la oxidación observado al homogeneizar la leche, debido a la modificación de la composición interfacial de los glóbulos y la formación de una capa protectora constituida por la caseína adsorbida (Frankel, 1998c). La interfase puede contener también moléculas capaces de atrapar radicales libres. Así, muchos emulsionantes (como los Tween) contienen moléculas de azúcares o aminoácidos particularmente efectivas atrapando radicales por su relativamente alta concentración en la interfase. En definitiva, la estabilidad oxidativa de una emulsión depende en gran medida del tipo, localización y concentración de emulsionante que puede quelar metales, inactivar radicales libres o formar una barrera física en la interfase (Donnelly y col., 1998; Berton-Carabin y col., 2014).

Las distintas moléculas presentes en la emulsión se distribuyen de acuerdo con su polaridad y actividad tensioactiva entre la fase lipídica, la fase acuosa y la interfase, y este fenómeno es de extraordinaria importancia en la efectividad de los antioxidantes. Frankel y colaboradores (Frankel, 2005) han realizado numerosos trabajos en este contexto y relacionado el fenómeno de la “paradoja polar” con la afinidad de los antioxidantes hacia las interfases aire/aceite en fase continua y agua/aceite en emulsión. De esta forma, en fase continua, los antioxidantes más hidrofílicos serían más efectivos por estar orientados hacia la interfase aire-aceite mientras que los antioxidantes lipofílicos serían menos protectores por permanecer disueltos en la fase oleosa, donde están presentes a bajas concentraciones. En cambio, en emulsiones, los antioxidantes lipofílicos se orientan hacia la interfase aceite-agua debido a su actividad tensioactiva y son más protectores que los hidrofílicos, que se disuelven y diluyen en la fase acuosa.

En relación con los azúcares se ha sugerido que el aumento de viscosidad que produce su adición a emulsiones alimentarias disminuye la concentración de oxígeno en la fase acuosa y por tanto su coeficiente de difusión. Además, los azúcares en concentraciones altas pueden atrapar radicales libres (Sims, 1994). Sin embargo, también pueden establecer enlaces con radicales y acelerar el proceso de oxidación (Yamauchi y col., 1988). La efectividad de las proteínas para retardar la oxidación en emulsiones depende de su partición entre la interfase y la fase acuosa, la composición de la interfase y la conformación. Aunque tienden a acumularse en la interfase, pueden estar presentes en la fase acuosa cuando se satura la interfase o bien cuando son desplazadas por ingredientes con mayor actividad interfacial. Su efecto antioxidante se ha atribuido a la capacidad de formación de membranas viscoelásticas capaces de restringir la penetración o difusión de iniciadores de la oxidación al interior de las gotas, y a su capacidad de atrapar radicales libres siendo así preferentemente oxidadas, como se ha observado con los péptidos cisteína y glutatión (Tong y col., 2000; Elias y col., 2008). Se ha observado que las principales proteínas de la leche, especialmente las caseínas, tienen capacidad antioxidante en emulsiones acuosas (Allen y Wierden, 1982a, 1982b).

Por último no hay que olvidar que los alimentos en emulsión, como la mayoría de los productos lácteos, contienen un gran número de ingredientes adicionales en la fase acuosa que pueden a su vez actuar como prooxidantes o antioxidantes dependiendo de sus propiedades químicas, sus condiciones e interacciones con los componentes lipídicos (Velasco y col., 2002).

Productos en polvo.

Los productos lácteos en polvo provienen del secado de emulsiones naturales como la leche (leche en polvo) o de emulsiones preparadas para emular la leche materna (fórmulas infantiles). En estos productos, los lípidos se encuentran en su mayor parte como gotículas o glóbulos rodeados de los demás componentes (proteínas, hidratos de carbono, etc.) en estado seco. Estas circunstancias añaden complejidad al estudio de la oxidación ya que otras variables pueden ejercer una influencia adicional, como la actividad del agua, el tamaño de

partícula, el tamaño de glóbulo lipídico y la distribución parcial de los lípidos en la superficie de las partículas y no en gotículas internas (Velasco y col., 2003; Vignolles y col., 2007; Barden y Decker, 2016).

Un aspecto de considerable interés en los productos lácteos en polvo es la facilidad del agua para producir cambios estructurales y afectar de una u otra forma la capacidad del oxígeno para alcanzar los componentes susceptibles de oxidación. Los lácteos en polvo son normalmente preparados por atomización, y se caracterizan por poseer a menudo una estructura metaestable que persiste en ausencia del efecto plastificador de la humedad o de temperaturas altas. Sin embargo, la adición de agua y/o el aumento de temperatura causa el fenómeno conocido como colapso, es decir, la disminución de volumen por aumento de la movilidad de componentes que da lugar a reorganizaciones de la estructura, pasando de un sistema en no equilibrio a un sistema en equilibrio termodinámico (Levine y Slade, 1990).

En la leche en polvo, la cristalización de la lactosa está relacionada con el fenómeno del colapso, durante el cual se libera agua desde la región cristalizada a la amorfa (Chuy y Labuza, 1994). La lactosa puede estar en estado cristalino o amorfo dependiendo del proceso de secado y las condiciones de humedad durante el almacenamiento. En leches en polvo, tras el secado por atomización, la lactosa está mayoritariamente en estado amorfo, y empieza a cristalizar a temperatura ambiente y actividades de agua de aproximadamente 0,40 (Buma, 1968). Algunos investigadores creen que el efecto de la actividad del agua en la oxidación lipídica en productos en polvo es indirecto y se explica mejor mediante la teoría de la transición vítrea de acuerdo con la cual la matriz polimérica de proteínas e hidratos de carbono que rodea a los lípidos puede estar en estado amorfo, facilitando su movilidad y reacción con oxígeno, o en estado vítreo, formando una estructura con mayor capacidad aislante y encapsulante porque hay menos espacio libre, estando éste ocupado por las cadenas de las macromoléculas de los polímeros. La temperatura de transición vítrea (Tg) determina cuándo ocurre el cambio de un estado a otro. Por ejemplo, para la lactosa, a 0, 1 y 4% de contenido en humedad, la Tg es 100, 70 y 45°C, respectivamente (Fritsch, 1994). De acuerdo con esta teoría, para aumentar la

estabilidad oxidativa, las formulaciones deben ser dirigidas a obtener matrices en el estado vítreo en las condiciones deseadas (Roos, 1996).

1.1.3. Métodos analíticos para la evaluación de la oxidación lipídica.

Se han desarrollado un gran número de métodos analíticos para evaluar el estado de oxidación de los aceites y lípidos de los alimentos (Frankel, 2005; Kamal-Eldin y Pokorny, 2005). Sin embargo, no existe un método universal que evalúe el estado de oxidación de forma objetiva y cuantitativa, de ahí que normalmente se recomiende el uso combinado de métodos (Barriuso y col., 2013).

1.1.3.1. Análisis sensorial.

El análisis sensorial es el más relacionado con la calidad de los lípidos de los alimentos y a veces permite la detección de olores y sabores que no pueden ser detectados por ningún método instrumental. Sin embargo, incluso con panelistas expertos, la reproducibilidad de los resultados es baja y por ello se recomienda utilizar complementariamente métodos químicos e instrumentales (Frankel, 2005).

1.1.3.2. Índices analíticos.

Índice de peróxidos.

Es una de las técnicas más utilizadas en la industria alimentaría por su sencillez y bajo coste. Es un índice de gran utilidad para evaluar la oxidación primaria por tratarse de una medida de los hidroperóxidos. Su principal limitación es que proporciona información errónea en muestras en estado de oxidación avanzado en las que un alto porcentaje de hidroperóxidos y peróxidos se han descompuesto dando productos secundarios de oxidación (Dobarganes y Velasco, 2002).

Índice de dienos conjugados.

Es un método indirecto para detectar hidroperóxidos formados durante la oxidación primaria basado en que el sistema de dobles enlaces conjugados se forma durante la formación de hidroperóxidos. Sin embargo, la relación entre formación de hidroperóxidos y formación de dienos conjugados no es siempre 1:1 debido a la presencia de hidroperóxidos monoenos y a la existencia de posteriores reacciones de ciclación y polimerización que pueden experimentar los hidroperóxidos conjugados. Además, otros compuestos, como los alcoholes, absorben a la misma longitud de onda (232 nm). Por tanto, puede decirse que el método es indicativo del proceso de oxidación más que de la formación de hidroperóxidos (Halliwell y Chirico, 1993).

Test del ácido tiobarbitúrico.

Se basa en la reacción entre el ácido tiobarbitúrico (TBA) y el malonaldehído o un precursor de éste. La reacción no es específica y otros compuestos de oxidación reaccionan también con el ácido tiobarbitúrico y por ello se les denominan junto al malonaldehído TBARS (conjunto de sustancias que reaccionan con el TBA) (Pokorný y col., 2005). La aplicación del método se limita a muestras de elevado grado de insaturación ya que sólo se detectan los productos de oxidación de ácidos grasos con 3 o más dobles enlaces. El método tiene muchos inconvenientes, como la interferencia de los productos de pardeamiento no enzimático y de degradación de proteínas y azúcares (St. Angelo, 1996).

1.1.3.3. Evaluación de compuestos de oxidación.

La evaluación de los compuestos de oxidación formados, ya sea compuestos específicos o grupos de compuestos, está basada fundamentalmente en técnicas cromatográficas. Éstas se han aplicado en el caso de compuestos específicos fundamentalmente a la cuantificación de compuestos volátiles (hidrocarburos y aldehidos) y, en menor medida, debido a su complejidad, a los compuestos de oxidación mayoritarios, los no volátiles (Thomas y col., 1991; Wilson y col., 1997),

aun cuando éstos últimos poseen un mayor interés desde el punto de vista nutricional ya que son los ingeridos con el alimento.

Hidroperóxidos.

La técnica más utilizada para el análisis de hidroperóxidos es la cromatografía líquida de alta resolución (HPLC) de adsorción en fase normal con detección UV. Existen numerosos trabajos que consiguen separar isómeros geométricos y de posición de monohidroperóxidos de ácidos grasos o sus productos tras ser reducidos, así como triglicéridos que contienen un resto acilo con un grupo hidroperóxido (Chan y Levett, 1977; Neff y Frankel, 1980; Frankel y col., 1990). La cromatografía HPLC sólo se ha aplicado hasta ahora para el análisis cualitativo de los principales productos de oxidación del linoleato de metilo (Mäkinen and Hopia, 2000) y linoleato de metilo conjugado (Hämäläinen y col., 2002). También se utiliza la cromatografía HPLC en fase reversa (C_{18}) para la separación de hidroperóxidos tras la reducción a derivados hidroxilo (Neff y col., 1992).

Compuestos volátiles.

La cromatografía gas-líquido (CGL) ha sido muy empleada para analizar aldehídos, alcoholes e hidrocarburos volátiles (Frankel, 2005). En la técnica de espacio de cabeza estático, la más sencilla y rápida, sólo se detectan compuestos presentes a concentraciones superiores a 0,1 mg/kg. Sin embargo, mediante el espacio de cabeza dinámico y el método de inyección directa se pueden detectar volátiles en cantidades traza (Rossell, 1994; Frankel, 1998b). A diferencia de lo que ocurre en la extracción estática, los volátiles no alcanzan el equilibrio entre la fase gas y la matriz, debido a que están siendo eliminados de la muestra continuamente, y no se requieren temperaturas tan elevadas. En los últimos años la técnica más utilizada para la extracción de volátiles es la microextracción en fase sólida (SPME) mediante una fibra recubierta de material adsorbente. La SPME ofrece una rápida transferencia de masa durante la extracción y la desorción, y facilita el manejo y la introducción directa en el cromatógrafo.

Compuestos monoméricos de oxidación secundaria.

Este grupo está constituido por compuestos monoméricos de oxidación secundaria, es decir, una compleja fracción que incluye funciones epóxidos, cetonas, aldehídos, alcoholes, hidroxiácidos, entre otros, y funciones polioxigenadas, cuya diversidad de estructuras se complica aún más en el caso de los triglicéridos, donde dichas funciones oxigenadas pueden estar en 1, 2 ó los 3 restos acilo. Dada la dificultad analítica, en triglicéridos sólo se ha abordado hasta el momento el análisis cualitativo de compuestos no volátiles presentes en triglicéridos oxidados utilizando la combinación de la cromatografía líquida (HPLC) y espectrometría de masas (Byrdwell y Neff, 2002; Sjövall y col., 2001, 2002; Suomela y col., 2004, 2005). Utilizando la cromatografía de gases, la identificación y cuantificación de los grupos de compuestos específicos presentes en la fracción monomérica de ácidos oxidados ya se ha abordado con éxito por nuestro grupo de investigación en aceites y grasas de fritura para los grupos aldehido-ácidos y epoxiácidos (Berdeaux y col., 1999a, 2002; Velasco y col., 2002, 2004a, 2005) y para los grupos de cetoácidos e hidroxiácidos (Marmesat y col., 2008).

Compuestos diméricos y poliméricos.

La cromatografía de exclusión molecular (HPSEC) es prácticamente la única técnica usada para la cuantificación de compuestos de polimerización (Wolff y col., 1991; IUPAC, 1992a). Aun cuando los dímeros y polímeros constituyen una compleja mezcla de compuestos, es posible obtener un pico definido para grupos de compuestos que difieren significativamente en peso molecular, como son los TG monoméricos, diméricos y poliméricos, con la columna y condiciones adecuadas. El análisis directo de polímeros mediante HPSEC puede ser aplicado a la evaluación de la calidad de muestras en que estos compuestos sean representativos de la alteración, como ocurre con los aceites y grasas usados en fritura y a la evaluación del estado de oxidación en aceites de elevado grado de insaturación (Dobarganes y Márquez-Ruiz, 2006). En condiciones de conservación, la cuantificación de los compuestos de polimerización es de gran utilidad ya que los estudios realizados con compuestos modelo, aceites y lípidos extraídos de alimentos han demostrado que la aparición de dímeros marca el

final del periodo de inducción y ocurre cuando se agotan los antioxidantes (Márquez-Ruiz y col., 2003b; Martín-Polvillo y col., 2004). Determinación de la pérdida de sustrato

Es una medida indirecta del grado de oxidación pues se basa en la cuantificación mediante patrón interno de los ésteres metílicos no alterados eluidos mediante CG en fase polar, y por diferencia se determina la cantidad de fracción no eluida, que está constituida por ésteres metílicos oxidados. Sólo es útil para estadíos avanzados de oxidación por su baja sensibilidad. La determinación del índice de polienos, utilizada en aceites de pescado, se basa en este análisis. Es el cociente entre la suma de las concentraciones de EPA y DHA y la del ácido palmítico (Lin y col., 1995).

Determinación cuantitativa de triglicéridos monómeros oxidados, dímeros y polímeros.

La combinación de la cromatografía de adsorción y exclusión molecular permite la cuantificación global de los triglicéridos que han sufrido oxidación en alguno de sus restos acilo, y su distribución en tres grupos principales de compuestos –triglicéridos monómeros oxidados, dímeros y polímeros- (Dobarganes y col., 1988; Márquez-Ruiz y col., 1996a; 1996b; Dobarganes y col., 2000; Márquez-Ruiz y Dobarganes, 2005; 2006; Dobarganes y Márquez-Ruiz, 2007). Básicamente consiste en la separación de la muestra de aceite o lípidos por polaridad (cromatografía de adsorción con gel de sílice) en triglicéridos no oxidados y una fracción concentrada de los compuestos de oxidación. Ésta última se analiza mediante cromatografía de exclusión y así pueden separarse grupos de compuestos de distinto peso molecular. Esta metodología ha sido estandarizada por la IUPAC y puede aplicarse tanto a aceites de fritura como a aceites o lípidos extraídos de alimentos oxidados (Dobarganes y col., 2000).

La cuantificación de los triglicéridos monómeros oxidados es de gran relevancia ya que este grupo incluye todas las moléculas monoméricas de triglicéridos con al menos un resto oxidado, ya sea con funciones hidroperóxido (compuestos de

oxidación primaria), o grupos oxigenados estables, por ejemplo, funciones epoxi-, ceto-, hidroxi-, etc. (compuestos de oxidación secundaria). Por tanto, su cuantificación constituye una medida global de los compuestos de oxidación no volátiles formados mediante reacciones de propagación y terminación. Se han obtenido excelentes correlaciones entre los triglicéridos monómeros oxidados y el índice de peróxidos durante el periodo de inducción (Márquez-Ruiz y col., 2007; Martín-Polvillo y col., 2004). La cuantificación de los triglicéridos dímeros y polímeros completa la información sobre el estado de oxidación ya que su formación indica el inicio de la fase de oxidación avanzada. La suma de triglicéridos monómeros oxidados, dímeros y polímeros es considerada la cantidad total de compuestos de oxidación no volátiles.

En estudios de oxidación lipídica esta metodología ha sido de gran utilidad para analizar la evolución de la oxidación en aceites y alimentos (Márquez-Ruiz y col., 1999; Martín-Polvillo y col., 2004), evaluar parámetros cinéticos (Márquez-Ruiz y col., 2003b; 2007) y detectar diferencias entre la oxidación en fase continua y discontinua de aceites microencapsulados (Márquez-Ruiz y col., 2003a; Velasco y col., 2000b, 2004b, 2006). La Figura 2. muestra un esquema de esta metodología. Como puede observarse, además pueden cuantificarse productos de hidrólisis, diacilgliceroles y ácidos grasos libres.

Análisis mediante técnicas espectroscópicas.

En los últimos años se han extendido las aplicaciones de métodos espectroscópicos para el análisis de la oxidación lipídica, fundamentalmente la Resonancia Magnética Nuclear (NMR) (Zamora y col., 2003; Hamalanien y Kamal-Eldin 2005, Guillén y Goicoechea 2007; 2009; Tyl y col., 2008) y la Espectroscopia Infrarroja de Transformada de Fourier (FTIR) (Guillén y Cabo, 2000; Guillén y col., 2004; Guillén y Goicoechea 2007). La principal ventaja de estas técnicas es su carácter no destructivo, el ahorro de reactivos y la reducción considerable del tiempo de análisis. Aunque sólo proporcionan información cualitativa relacionada con las funciones oxigenadas encontradas, en estudios

realizados con FTIR se ha encontrado una estrecha correlación entre valores de índice de peróxidos en aceites oxidados con los valores de las frecuencias de determinadas bandas del espectro infrarrojo (Guillén y Cabo, 2000). Así mismo, en aplicaciones de NMR de 13C se ha comprobado que existe una buena correlación entre los datos obtenidos mediante las señales asignadas a los triglicéridos oxidados en NMR y las cantidades de triglicéridos oxidados determinadas por cromatografías de adsorción y exclusión (Zamora y col., 2003).

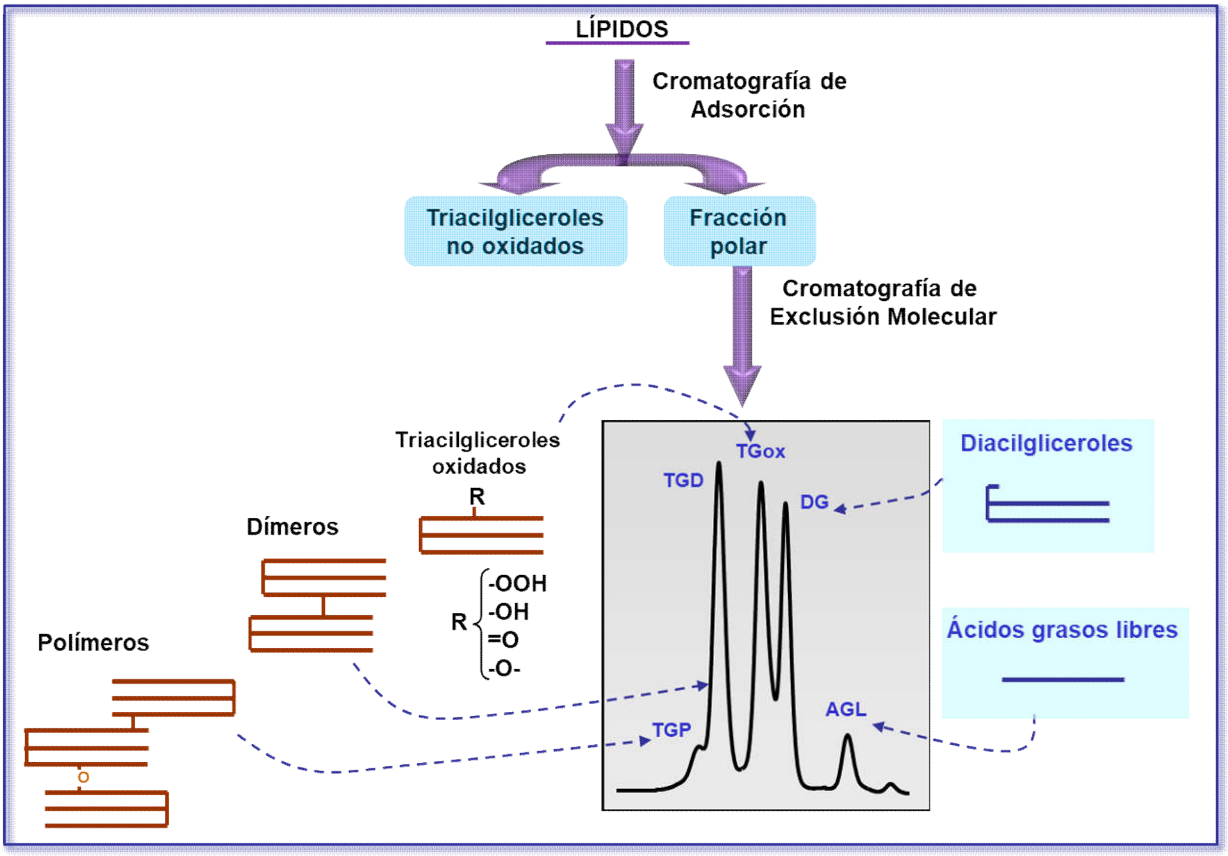

Figura 2.- Metodología utilizada para la determinación cuantitativa de los compuestos de oxidación no volátiles totales y su distribución en triacilgliceroles monómeros oxidados, dímeros y polímeros.

1.1.3.4. Determinación de la estabilidad oxidativa mediante métodos acelerados.

La estabilidad oxidativa se define como la resistencia a la oxidación lipídica y los métodos para su determinación se basan en acelerar el desarrollo oxidativo, normalmente mediante un aumento de la temperatura o la concentración de oxígeno, de forma que los resultados obtenidos ayuden a predecir el tiempo de vida útil del aceite o alimento. Estos métodos son esenciales para la evaluación de la actividad antioxidante en los aceites y pueden también aplicarse a alimentos (Rossell 1992, 1994; Verleyen y col., 2005; Frankel, 2005). El curso de la oxidación normalmente procede lentamente en la primera fase y experimenta un cambio repentino en la velocidad de oxidación después de un cierto periodo de tiempo, llamado periodo de inducción (PI). El PI se mide, por tanto, por estimación del punto de máxima curvatura o la intersección de las dos líneas tangentes a ambas ramas de la curva de oxidación. Los métodos que más se utilizan actualmente se resumen a continuación:

Método Oxidograph

Se basa en la determinación de la absorción de oxígeno en muestras calentadas a 100ºC, mediante la medida del descenso de presión de oxígeno, es un aparato automatizado y se pueden ensayar alimentos y antioxidantes volátiles.

Método OSI (índice de estabilidad oxidativa) o Rancimat

Consiste en oxidar las muestras con aire a flujo constante y temperatura constante que suele fijarse entre 80-140°C. Los compuestos volátiles que se originan, fundamentalmente ácidos orgánicos de cadena corta, son detectados por conductividad. El test OSI es un método oficial AOCS (Läubli y Bruttel, 1986).

Otros métodos

La calorimetría diferencial de barrido (DSC) y la espectroscopia de spin electrónico (ESR) pueden ser aplicadas para la determinación de la estabilidad oxidativa. La DSC normalmente se utiliza a temperaturas entre 100 y 140ºC con flujo de oxígeno constante (Tan y col., 2002). En el caso de la ESR, la estabilidad oxidativa se define como la resistencia de los lípidos a formar radicales a temperatura moderada (50-80ºC) y, a diferencia de la mayoría de los métodos,

que consideran el inicio de la fase de oxidación avanzada como el punto final del test, se basa en la medida de los radicales que se forman durante las fases más iniciales de la oxidación (Velasco y col., 2004a,b).

1.2. Oxidación lipídica en leche y productos lácteos convencionales.

Los lípidos de la leche, constituidos aproximadamente por un 98% de triglicéridos, se encuentran en estado natural formando glóbulos de grasa. La membrana del glóbulo graso está compuesta por proteínas (40%), fosfolípidos (30%), que incluyen lipoproteínas y glicoproteínas, glicéridos (14%), cerebrósidos (3%), colesterol (2%) y enzimas. La composición en ácidos grasos es aproximadamente de un 63-69% ácidos grasos saturados (24% palmítico, 13% esteárico), 27-34% monoinsaturados (25% oleico), y 2,5-3% poliinsaturados (2% linoleico) (Jensen, 2002). La membrana del glóbulo graso es el principal sitio donde ocurre la oxidación lipídica ya que contiene trazas de cobre y hierro, y su estructura es dinámica e inestable a los tratamientos térmicos. La oxidación se produce inicialmente en la fracción poliinsaturada asociada a los fosfolípidos que se encuentran en la membrana del glóbulo graso seguida de la oxidación en los triglicéridos. Aunque en la leche líquida la fracción de fosfolípidos es más susceptible a la oxidación que la fracción de triglicéridos, en la leche en polvo ocurre lo contrario y los fosfolípidos actúan como antioxidantes (Duncan y Webster, 2010).

Aunque la oxidación lipídica no es el principal problema de calidad de la leche, mucho más susceptible a alteraciones microbiológicas y reacciones de pardeamiento (Mortensen y col., 2010), los compuestos volátiles de oxidación de la leche producen un aroma indeseable, el cual es percibido en leche almacenada a muy bajos niveles de oxidación, habitualmente con un índice de peróxidos menor de 1 (Frankel, 2005). Esto se debe a que los compuestos volátiles producidos en la oxidación se volatilizan más rápidamente en un medio acuoso como la matriz de la leche. La percepción de los compuestos carbonílicos volátiles generados en la oxidación lipídica son detectables a concentraciones extremadamente bajas (partes por billón) y resultan más

evidentes en la leche, la nata y la mantequilla porque son alimentos de aroma suave.

La catálisis metálica está reconocida como el factor más importante en la aceleración de la oxidación lipídica en los productos lácteos. Aunque el cobre se encuentra de forma natural en la leche a menor concentración (20-40 µg/l) que el hierro (100-250 µg/l), es el catalizador más relevante en el desarrollo de volátiles de oxidación. Estos metales se encuentran en complejos proteínicos asociados a la membrana del glóbulo graso. El mecanismo más aceptado de oxidación lipídica en la leche incluye la acción catalítica de las lipoproteínas con cobre asociado. Las metaloproteínas actúan como catalizador de la oxidación en presencia de oxígeno y de sistemas redox que incluyen al ácido ascórbico.

El segundo factor más importante que determina la susceptibilidad oxidativa de la leche es la integridad de la membrana, la cual puede ser afectada por el procesamiento industrial (O'Connor y O'Brien, 1995).

El ácido ascórbico también está relacionado con el desarrollo de compuestos volátiles de oxidación en la leche, donde se encuentra en concentraciones de 10-20 mg/l. La concentración de ácido ascórbico en la leche está determinada por la alimentación animal y las condiciones de almacenamiento, ya que durante el almacenamiento de la leche va disminuyendo hasta su agotamiento por el consumo del oxígeno disuelto. La acción catalítica del cobre se incrementa en presencia de ácido ascórbico. Sin embargo, cuando la concentración de ácido ascórbico es alta (50-200mg/l), inhibe eficazmente el desarrollo de aromas de oxidación. Por tanto, la combinación de ascórbico y cobre puede tener efectos prooxidantes a bajos niveles de ascorbato o efecto antioxidante a altas concentraciones de ascorbato. Existen varias teorías para explicar este fenómeno, por ejemplo que se favorezca la conversión de los iones cúpricos a estado cuproso mas activo, disminuir la oxidación lipídica por la oxidación preferente del ácido ascórbico y la depleción del oxígeno disponible, o por la reducción de los hidroperóxidos mediante el ascorbato en alcoholes alílicos estables. Sin embargo, las reacciones químicas por las cuales se produce esta paradoja todavía no están claras.

Otros agentes reductores como los tioles también tienen efecto pro y antioxidante en presencia de cobre ya que su unión con cobre y hierro influye en su actividad catalítica. La distribución de los metales en diferentes productos lácteos y los efectos del procesado sobre su acción catalítica está influenciada por numerosos factores los cuales todavía no están totalmente esclarecidos.

La suplementación de los productos lácteos con hierro disminuye notablemente la vida útil de estos productos, especialmente en las fórmulas infantiles. La oxidación lipídica en productos con hierro añadido se puede controlar utilizando lactoferrina, una glicoproteína que se encuentra de forma natural en la leche y la cual se satura con hierro (Satué-Gracia y col., 2000).

El α-tocoferol es el único antioxidante natural de la leche, su concentración media es de 25 μg/g en la grasa láctea y 44 μg/g en la membrana del glóbulo graso. Su contenido varía en función de la alimentación del animal y de la época del año. La estabilidad oxidativa de la leche depende de la concentración de α-tocoferol, especialmente en la membrana del glóbulo graso. La adición directa de α-tocoferol a la leche es más eficaz para controlar la oxidación lipídica que administrar una alimentación rica en este antioxidante a los animales. La leche también está protegida frente a la oxidación por la caseína y otras proteínas capaces de unirse al cobre y al hierro e inactivarlos. Otro mecanismo sugerido para mejorar la estabilidad oxidativa es la homogenización de la leche, lo cual distribuye las caseínas por la membrana del glóbulo graso formando una capa protectora en la interfase agua-aceite. El tratamiento térmico de la leche también incrementa su estabilidad oxidativa ya que activa los grupos sulfidrilos de las proteínas.

La oxidación lipídica durante el procesado y almacenamiento de la leche y los derivados lácteos está influida por los prooxidantes y los antioxidantes y el oxigeno contenido en el producto final. Factores externos como la manipulación, agitación, almacenamiento, temperatura, exposición a la luz y contaminación por metales o microorganismos tienen una influencia importante en el desarrollo oxidativo. El oxígeno contenido en la leche pasteurizada es aproximadamente 9 mg/l a temperatura ambiente, y se ha mostrado que el almacenamiento en

oscuridad consume 4,1 mg/l después de 6 días. Las bacterias y la oxidación del ácido ascórbico consumen mucho más oxigeno que la oxidación de los grupos sulfídrilos o la autoxidación. El desarrollo de aromas de oxidación en productos lácteos se inhibe eficazmente retirando el oxígeno disuelto por desaireación. La vida útil de los productos lácteos aumenta considerablemente mediante tratamientos de vacío o reemplazando el oxígeno por un gas inerte. La pasteurización de la leche incrementa la susceptibilidad a la oxidación al aumentar la concentración de cobre en la fase grasa. Sin embargo, si la temperatura alcanzada es muy elevada se forman compuestos reducidos de los grupos sulfidrilos con actividad antioxidante. La homogenización de la leche rompe el glóbulo graso natural y forma glóbulos grasos con una membrana de caseínas (93%) y proteínas del suero lácteo. Este proceso retarda la oxidación lipídica y el desarrollo de aromas de oxidación de forma efectiva. Aunque se han publicado numerosos trabajos en esta área, los cambios estructurales de la membrana del glóbulo graso resultantes de la homogenización todavía no están claros.

Las leches en polvo, además de ser comercializadas para su consumo directo, son ingredientes muy utilizados en la elaboración de gran variedad de alimentos, como por ejemplo el chocolate, productos horneados, alimentos infantiles e incluso son empleadas en nutrición animal. Se obtienen normalmente por atomización de la leche entera o parcialmente desnatada. Como se ha detallado en el apartado 1.1.2.7. (productos en polvo), el estudio de sus propiedades fisicoquímicas es de enorme relevancia para mejorar su estabilidad oxidativa (Vega y Roos, 2006; Vignolles y col., 2007).

1.3.- Productos lácteos enriquecidos con ácidos grasos funcionales: antecedentes de estudios de oxidación.

1.3.1.- Productos lácteos enriquecidos con ácidos grasos poliinsaturados ω-3 de cadena larga.

Los efectos beneficiosos de los ácidos grasos poliinsaturados (AGPI) ω-3 de cadena larga (ácido eicosapentaenoico y ácido docosahexaenoico), mayoritarios en aceites de pescado y algas, han sido ampliamente demostrados, y destaca su papel en la prevención de las enfermedades cardiovasculares e inflamatorias (Shahidi, 2015). Además, tienen efectos positivos en la prevención y desarrollo de procesos cancerígenos, en procesos alérgicos y en la capacidad cognitiva (Ruxton y col., 2007).

Los AGPI esenciales, es decir, los ácidos linoleico y α-linolénico, sólo proceden de la dieta ya que los tejidos de los mamíferos carecen de los enzimas capaces de sintetizarlos. A partir de ambos se sintetizan por desaturación y elongación los AGPI de cadena larga. Existen cuatro series de AGPI, los ω-3, formados a partir del ácido α-linolénico, los ω-6 a partir del ácido linoleico, los ω-7 a partir del ácido palmítico y los ω-9 a partir del ácido oleico. La conversión del ácido α-linolénico a ácido eicosapentanoico (EPA, C20:5 ω-3) y docosahexanoico (DHA, C22:6 ω-3) es relativamente lenta y ocurre sólo en algunos tejidos. Como los ácidos grasos ω-6 y ω-3 compiten por el mismo sistema enzimático, si la dieta es mayoritaria en ácido linoleico, como ocurre en la mayoría de los países occidentales, la síntesis de AGPI ω-3 de cadena larga se ve disminuida y por ello es recomendable aumentar el aporte de ácido α -linolénico o directamente AGPI ω-3 de cadena larga en la dieta (Trautwein, 2001).

Debido al descenso generalizado en el consumo de productos de la pesca, existe una creciente tendencia a adicionar AGPI ω-3 de cadena larga a una gran variedad de alimentos (productos lácteos, margarinas, productos horneados, zumos, salsas, preparados dietéticos, alimentos infantiles, etc) (Garg y col., 2006). En productos lácteos, se pueden aumentar los niveles de AGPI ω-3 alternativamente mediante la inclusión de aceites de pescado o algas en la dieta

de los rumiantes (Dave y col., 2002) o el uso de determinadas bacterias, en el caso de las leches fermentadas y yogures (Khurana y Kanawjia, 2007).

La oxidación lipídica es un problema muy importante en la preparación y comercialización de los productos lácteos enriquecidos con AGPI ω-3. Los olores resultantes poseen un umbral bajo y son particularmente desagradables y, por tanto, la presencia de compuestos de oxidación volátiles, incluso a muy bajas concentraciones, afecta significativamente a la calidad sensorial de dichos productos lácteos. Ésta es la principal razón por la cual la mayoría de los estudios que examinan la estabilidad oxidativa de los productos lácteos enriquecidos con AGPI ω-3 se hayan enfocado exclusivamente a la determinación de volátiles y el análisis sensorial.

La evaluación sensorial proporciona información insustituible sobre la aceptabilidad de los alimentos pero exige un panel de expertos muy especializados, muestra generalmente baja reproducibilidad y se requieren cantidades relativamente grandes de muestras. Es necesario, por tanto, utilizar métodos analíticos instrumentales para complementar y ampliar la información aportada por la evaluación sensorial.

La determinación de los compuestos de oxidación volátiles en productos lácteos enriquecidos con AGPI ω-3 proporciona normalmente resultados concordantes con el índice de peróxido y el análisis sensorial (Jiménez-Álvarez y col., 2008) aunque el método puede mostrar falta de sensibilidad (Let y col., 2005). Aun así, la determinación exclusiva de compuestos de oxidación volátiles no es suficiente desde el punto de vista nutricional porque es imprescindible conocer la cantidad de compuestos de oxidación no volátiles formados que van a ser consumidos.

El índice de peróxidos es el método más utilizado para controlar la oxidación lipídica en los productos lácteos funcionales. Como ya se ha comentado, es una medida del contenido de compuestos de oxidación primaria, los hidroperóxidos, que se forman en la fase temprana de oxidación. Es necesario extraer la grasa del alimento tomar precauciones al evaporar el disolvente para evitar la descomposición de los hidroperóxidos, y es posible que se extraigan

conjuntamente compuestos reductores que pueden disminuir el índice de peróxido (Velasco y col., 2008). Otro inconveniente en lípidos muy insaturados es la rápida descomposición de los hidroperóxidos por lo que el índice de peróxidos empieza a disminuir en etapas tempranas de oxidación y no refleja el estado oxidativo real (Márquez-Ruiz y col., 2000; Velasco y col., 2006).

El grupo de investigación de la Dra. Jacobsen ha realizado, con diferencia, el mayor número de trabajos en el contexto de la oxidación lipídica en productos lácteos enriquecidos con AGPI ω-3 (Nielsen y col., 2007; Jacobsen y col., 2008; Let y col., 2003, 2004, 2005a, 2005b, 2007a, 2007b; Sorensen y col., 2007; Lu y col., 2013; Saga y col., 2013; Jacobsen, 2010, 2015). El procedimiento generalmente llevado a cabo en estos estudios consiste en la adición de aceite de pescado a leche comercial, seguido de homogenización para obtener emulsiones que contengan 1% de grasa láctea y entre 0,25 y 1,5% de aceite de pescado. El aceite de pescado utilizado mostró siempre niveles aceptables de tocoferoles y muy bajos niveles de índice de peróxidos, las muestras fueron normalmente almacenadas a muy bajas temperaturas y los métodos usados fueron el análisis sensorial combinado con la determinación de volátiles, junto con el índice de peróxidos o la pérdida de antioxidantes. En general, los resultados obtenidos por este grupo mostraron niveles de oxidación muy bajos (Let y col. 2004; 2005a, 2005b), sólo se detectó oxidación en productos lácteos enriquecidos con aceite de pescado cuando el aceite estaba exento de antioxidantes o las condiciones de homogenización fueron desfavorables (Let y col., 2005; Sorensen y col., 2007) y el yogur fue el producto lácteo que experimentó el menor desarrollo de rancidez (Let y col., 2007a; Lu y col., 2013). Así mismo, otros autores han encontrado que leches fermentadas enriquecidas con AGPI ω-3 no experimentaron cambios en composición de ácidos grasos ni en el perfil de volátiles durante su almacenamiento (Luna y col., 2004). En relación con el efecto de las condiciones de homogenización y la composición proteica, los resultados indicaron que la composición de la interfase aceite-agua ejercía más influencia en la oxidación que el tamaño de glóbulo (Let y col., 2007b), siendo más estables las combinaciones con más β-lactoglobulina y menos caseína (Sorensen y col., 2007). En cuanto a los métodos utilizados, el grupo de Jacobsen concluye que el análisis sensorial es más sensible que el

índice de peróxidos y que la determinación de volátiles aunque éstos dos últimos muestran buena correlación.

Estudios realizados por otros investigadores, enfocados a evaluar la influencia de la fortificación con aceite de pescado en la calidad sensorial de yogures, quesos frescos y procesados, mantequilla y nata, muestran que el nivel de fortificación en productos lácteos es limitado, y los productos que admiten los niveles más altos son los sólidos y con gran contenido en grasa (quesos frescos para untar, quesos procesados y mantequilla) (Kolanowski y Weibbrodt, 2007). Otros estudios, no dirigidos a evaluar la oxidación lipídica, realizados en yogures y leches fermentadas, han analizado la influencia de la adición de AGPI ω-3 en la textura, el sabor y el olor (Barrantes y col., 1994a, 1994b; Martín-Diana y col., 2004).

Hay que destacar que los estudios publicados hasta el momento sobre oxidación lipídica en productos lácteos funcionales enriquecidos con AGPI ω-3 se han realizado prácticamente en su totalidad en muestras a las que se adiciona AGPI ω-3 durante el desarrollo experimental y no en muestras comerciales, donde la influencia del procesado y la comercialización y almacenamiento se desconoce.

1.3.2.- Fórmulas infantiles.

Las fórmulas infantiles son productos destinados a satisfacer de manera total o parcial las demandas nutritivas de los lactantes durante los primeros meses de vida, en sustitución de la leche materna. Se pueden distinguir tres grupos de fórmulas: de inicio (hasta los 6 meses), de continuación (de 6 a 12 meses) y de crecimiento (a partir del primer año de vida). La alimentación durante los primeros meses de vida tiene una influencia decisiva, no sólo en las fases tempranas del desarrollo del niño sino también en su salud a largo plazo. Por ello, la incorporación de nuevos ingredientes funcionales a las fórmulas infantiles ha dado lugar a una gran diversidad de productos. Algunos ingredientes de interés en la alimentación del lactante son los ácidos grasos poliinsaturados, oligosacáridos con efecto prebiótico, probióticos, carnitina y taurina, entre otros.

Las fórmulas infantiles se encuentran en el mercado como concentrados líquidos, como preparados líquidos listos para tomar y, sobre todo, como productos en polvo. Las fórmulas infantiles en polvo se preparan normalmente a partir de leche de vaca que ha sido desnatada total o parcialmente y diferentes aceites vegetales, además de otros hidratos de carbono y proteínas y oligoelementos (Thompkinson y Kharb, 2007; Joeckel y Phillips, 2009); y posteriormente se realiza su secado para obtener el producto en polvo, siendo la atomización el método más empleado (Joeckel y Phillips, 2009; Thompkinson y Kharb, 2007). Con el objetivo de aumentar la calidad nutricional y mejorar el sabor, se eliminan algunas proteínas y se adicionan diferentes hidratos de carbono, normalmente lactosa. Las proteínas más abundantemente presentes en estos productos son las derivadas de la leche de vaca como caseínas, combinaciones de caseínas y proteínas del suero o concentrados de hidrolizados de proteínas del suero. Además de la lactosa, también se emplean habitualmente en las fórmulas infantiles maltodextrinas, sacarosa y jarabe de maíz como hidratos de carbono. Las fórmulas infantiles contienen combinaciones de aceites y grasas adecuadas para obtener una proporción de ácidos grasos saturados e insaturados similar a la que existe en la leche materna, especialmente en ácido linoleico y α-linolénico. La leche materna contiene aproximadamente el 44% de ácidos grasos saturados, 17% de ácidos grasos poliinsaturados y 39% de ácidos grasos monoinsaturados. Actualmente, las fórmulas infantiles contienen mayor cantidad de aceites vegetales que de grasa láctea y los más utilizados son girasol alto oleico, soja, palma y oleína de palma.

Una tendencia creciente es la incorporación de AGPI ω-3 de cadena larga porque son esenciales para el desarrollo visual y neuronal del lactante, y especialmente beneficiosos en los tres primeros meses de vida (Fleith y Clandinin, 2005). Con este objetivo se adicionan aceites de pescado o aceites derivados de microalgas, como fuentes de C20:4 ω-6 (ácido araquidónico, AA) y C22:6 ω-3 (ácido docosahexaenoico, DHA). Otra fuente de AGPI ω-3 para fórmulas infantiles son los fosfolípidos de yema de huevo producidos mediante la introducción de pescado en la alimentación de pollos (Trautwein, 2001). Además, es necesario adicionar a las fórmulas infantiles vitaminas y minerales para cubrir los requerimientos nutricionales. Como consecuencia, las fórmulas infantiles

contienen normalmente una proporción relativamente alta de ácidos grasos insaturados, susceptibles a la oxidación, y de minerales prooxidantes como el hierro; y es fundamental estudiar las modificaciones químicas que pueden ocurrir durante su preparación y conservación para mantener su calidad nutricional y controlar la formación de lípidos oxidados potencialmente tóxicos (Nasirpour y col., 2006).

La oxidación de los lípidos de las fórmulas infantiles durante su almacenamiento ha sido estudiada en diversos trabajos (De la Presa-Owens, 1995; Angulo y col., 1998; Manglano y col., 2005; García-Llatas y col., 2006; Romeu-Nadal y col., 2007; Rodríguez-Alcalá y col., 2007; Chavez-Serrín y col., 2008; Velasco y col., 2008; Michalsky y col., 2008) pero la complejidad composicional de estos productos y del desarrollo de la oxidación en estos sistemas lipídicos heterofásicos ha dado lugar a resultados inconsistentes (De la Presa-Owens, 1995; Angulo y col., 1998; Manglano y col., 2005; García-Llatas y col., 2006; Chávez-Servín y col., 2008). Concretamente, una de las causas de la incongruencia de los resultados obtenidos es la influencia de los distintos procedimientos utilizados en la extracción de los lípidos y la selección de métodos sólo aplicables a estadíos específicos del proceso de oxidación (Velasco y col., 2008).

En fórmulas infantiles suplementadas con AGPI ω-3 se han encontrado cambios significativos en el contenido de estos ácidos grasos y en volátiles durante su almacenamiento a 37°C (Romeu-Nadal y col., 2007). Por otra parte, se han reportado importantes pérdidas de tocoferoles tanto en fórmulas suplementadas con sulfato o lactato de hierro a 22-25°C (Miguel y col., 2004; Chávez-Servín y col., 2008), mientras que en otros estudios no se han detectado cambios a 20, 30 o 37°C (Albalá-Hurtado, 2000). Es importante destacar que en ninguno de estos estudios se realizó una extracción separada de la fracción de lípidos superficiales, que está más expuesta al oxígeno del aire y por tanto es más susceptible a la oxidación. La importancia de analizar esta fracción ya ha sido enfatizada en el caso de la leche en polvo (Vignolles y col., 2007). En este tipo de alimentos en polvo donde la distribución de los lípidos es heterogénea puede ocurrir que el nivel de oxidación global sea todavía muy bajo y el alimento

muestre ya rancidez debido a que la rápida oxidación de una fracción de lípidos superficiales muy minoritaria (Velasco y col., 2006).

1.3.3. Productos lácteos enriquecidos con ácido linoleico conjugado (CLA).

Ácido linoleico conjugado (CLA) es un acrónimo que engloba a un conjunto de isómeros geométricos y posicionales del ácido linoleico conjugado que se encuentran principalmente, y de forma minoritaria, en la carne procedente de rumiantes (como resultado de procesos de biohidrogenación en el rumen) y en los productos lácteos (con una concentración de 0,6 a 1,6% de la composición de ácidos grasos) (Fristche y col., 1999). Los isómeros con conocido efecto fisiológico son el ácido linoleico *cis*-9, *trans*-11 y el ácido linoleico *trans*-10, *cis*-12 (Pariza y col., 2000; Yang y col., 2015). Se han atribuido propiedades anticarcinogénicas al isómero *cis*-9, *trans*-11 ácido linoleico, que es el isómero más abundante en la naturaleza, también conocido como ácido ruménico (Masso-Welch y col., 2004), mientras que el *trans*-10, *cis*-12 ácido linoleico contribuye a la pérdida de peso corporal y aumento de la masa muscular (Malpuech-Bruguere y col., 2004; Whigham y col., 2007, Raff y col., 2009), bien por la inhibición de la lipogénesis (formación de tejido adiposo) como por el potencial estímulo que puedan ejercer sobre la lipolisis (transformación de grasas en energía). Las estimaciones de ingesta diaria de CLA varían entre 212 mg a 1,5 g al día (Fritsche y col., 1999) mientras que se ha propuesto que para conseguir los beneficios óptimos para la salud la ingesta debiera ser de 3 g al día para un adulto de 70 kg (Ip y col., 1994), y ello justifica su creciente utilización en suplementos dietéticos y alimentos, sobre todo en productos lácteos.

Aunque existen diferentes métodos para enriquecer los productos lácteos comerciales en CLA, como la modificación de las dietas de los rumiantes con suplementos ricos en ácidos grasos poliinsaturados (Stanton y col., 2003; Khanal y Olson, 2004), el procedimiento más efectivo y más utilizado consiste en la adición directa de aceites ricos en CLA (Rodríguez-Álcala y Fontecha, 2007; García-Martínez y Márquez-Ruiz, 2009). La mayoría de los aceites ricos en CLA se obtienen a partir de aceites vegetales en los que el ácido linoleico es mayoritario, por ejemplo aceites de cártamo o girasol, mediante procesos

químicos (interesterificación) que convierten el ácido linoleico en CLA (aprox. > 80%), una mezcla a partes iguales de los dos isómeros biológicamente activos (*cis*-9, *trans*-11 y *trans*-10*, cis*-12) más una pequeña proporción de otros isómeros (Ohr, 2005). En el caso de las leches fermentadas, se consigue eficientemente el aumento de CLA utilizando determinadas bacterias (Kim y Liu, 2002).

Los estudios de estabilidad realizados en productos lácteos enriquecidos con CLA no muestran, en general, diferencias en parámetros organolépticos de calidad (Bisig y col., 2007). Concretamente en los lácteos enriquecidos a través de suplementos en las dietas de rumiantes, no se han encontrado efectos o muy leves. Por ejemplo, no se detectaron diferencias en aroma o sabor en leches y quesos cuando se enriquecieron con CLA mediante suplementación de la dieta con aceite de pescado (Avramis y col., 2003; Jones y col., 2005) ni en mantequilla rica en CLA obtenida a partir de leche de vacas alimentadas con semillas de girasol después de 6 semanas de almacenamiento (Mallia y col., 2008). Lynch y colaboradores no encontraron cambios en la composición de ácidos grasos en leche rica en CLA expuesta a la luz durante 14 días (Lynch y col., 2005), y González y colaboradores tampoco encontraron diferencias en el índice de peróxido cuando prepararon mantequilla y helados con leche rica en CLA (González y col., 2003).

En relación a las características organolépticas de los productos a los que se adiciona aceites ricos en CLA, donde el contenido en CLA es mucho más elevado, no se ha observado disminución de la calidad en productos lácteos con CLA mediante análisis sensoriales (Campbell y col., 2003) ni se han encontrado diferencias en la formación de hexanal (Jiménez y col., 2008). En un estudio donde se examinaron los cambios en la composición de ácidos grasos de 6 productos comerciales con CLA (con 1,2 g de CLA por ración) durante su procesado y tras 10 semanas de refrigeración, se observaron disminuciones significativas de algunos de los isómeros minoritarios y ligero pero significativo aumento de isómeros *trans, trans*, especialmente en leche en polvo (Rodríguez-Alcalá y Fontecha, 2007). Los isómeros *trans, trans* se han encontrado como componentes menores tanto en el CLA presente de forma natural en productos

lácteos (Fritsche y Steinhart, 1998) como en las mezclas sintéticas de CLA (Adlof y col., 2001) y su aumento de asocia con los tratamientos a elevada temperatura (Juaneda y col., 2003).

La evaluación de la oxidación del CLA, así como de los PUFA ω-3, se realiza frecuentemente mediante la determinación de la pérdida de sustrato, es decir, calculando la disminución de dichos ácidos grasos en la composición de ésteres metílicos analizados por cromatografía de gases (Luna y col., 2004; Lynch y col., 2005; Rodríguez-Alcalá y Fontecha, 2007). Además, la composición de ésteres metílicos es utilizada rutinariamente por la industria como control del nivel de ácidos grasos funcionales en los productos lácteos enriquecidos. Sin embargo, se ha demostrado que muestra menor sensibilidad y exactitud que la determinación directa de los compuestos de oxidación formados (Márquez-Ruiz y col., 2007; Luna y col., 2007). Aún así, mediante esta determinación se han reportado pérdidas en leche enriquecida con CLA de hasta un 10% después de pasteurización y pérdidas adicionales de hasta el 6% durante su conservación a temperaturas de refrigeración (Campbell y col., 2003), así como pérdidas del 22% en leche enriquecida con CLA después del tratamiento de esterilización UHT (Martínez-Monteagudo y col., 2015).

La evaluación del grado de oxidación del CLA es compleja, como consecuencia de que las rutas de oxidación del CLA no han sido claramente establecidas (Yurawecz y col., 1997, 2003; Eulitz y col., 1999; Brimberg y Kamal-Eldin, 2003; Luna y col., 2007). En contraste con lo que ocurre durante las primeras fases de la oxidación del ácido linoleico más abundante, no conjugado, 9 cis, 12 trans, la formación de hidroperóxidos en CLA es mucho más baja. Por tanto, los métodos utilizados normalmente, el índice de peróxidos y el TBARS, pueden no ser indicativos del nivel de oxidación en productos enriquecidos con CLA.

La efectividad de los antioxidantes para proteger al CLA de la oxidación ha sido abordada sólo en 4 estudios. El α-tocoferol ha mostrado menor protección que los antioxidantes sintéticos TBHQ, BHA, BHT y ácido gálico (Lee y col., 2003) y, comparado con sus homólogos, se ha observado mayor efectividad de los homólogos δ- y γ-, y muy baja para el α-, en estudios a 50°C y mediante

determinaciones de índice de peróxidos y TBARS (Ko y col., 2010). En estos dos estudios se evaluaron muestras de CLA en forma de ácido graso libre. El único trabajo donde se utiliza CLA esterificado en triglicéridos, es decir, en estructuras químicas similares a los aceites ricos en CLA utilizados en productos lácteos funcionales, es en nuestro conocimiento el desarrollado por Tsuzuki y colaboradores (2004). Los autores concluyeron, en base a medidas de TBARS y pérdida de sustrato, que el α-tocoferol aumentaba la estabilidad de forma similar en triglicéridos ricos en ácido linoleico no conjugado que en los ricos en ácido linoleico conjugado. Encontraron que la pérdida de sustrato en el caso de los sistemas con CLA fue significativamente más elevada que la indicada por la formación de TBARS. Esta aparente contradicción puede deberse a la formación de polímeros, como nuestro grupo ha observado en estudios cinéticos de oxidación en compuestos modelo (Luna y col., 2007).

Además de la adición de antioxidantes, otra estrategia que puede abordarse para proteger al CLA de la oxidación es la microencapsulación. Esta tecnología ya se aplica para obtener ingredientes de AGPI ω-3 y adicionarlos a diversos alimentos incluidos determinados productos lácteos (Encina y col., 2016; Bakry y col., 2016). De forma resumida, la microencapsulación de aceites y lípidos consiste en la preparación de una emulsión de aceite en agua, conteniendo las proteínas y/o hidratos de carbono que constituirán la matriz encapsulante, y su posterior secado, normalmente por atomización, cuyo resultado es un producto en polvo. Entre los hidratos de carbono más utilizados cabe destacar la lactosa, de sabor y olor neutros, alta solubilidad y baja viscosidad (Vega y Roos, 2006). Se usa normalmente en combinación con proteínas de la leche. Otros hidratos de carbono utilizados son las ciclodextrinas, sacarosa, maltodextrinas, almidón de maíz, gomas y éteres de celulosa. Entre las proteínas, de gran capacidad encapsulante debido a sus propiedades emulsificantes, espumantes, de hidratación y de gelificación, destacan las proteínas de la leche, es decir, las caseínas y las proteínas del suero de la leche (Augustin y col., 2010). Las caseínas y caseinatos poseen propiedades tensioactivas que los convierten en agentes encapsulantes de alto poder emulsionante, y entre ellos el caseinato sódico destaca como el más utilizado (Vega y Roos, 2006). Existen 4 tipos de caseínas (α_{s1}, α_{s2}, β, κ), que constituyen aproximadamente el 38, 10, 36 y 12%

de la fracción caseínica, y todas contienen un alto nivel de prolina, que evita la formación de estructuras secundarias haciéndolas así más estables frente a la desnaturalización y contribuye a su elevada actividad tensioactiva, y de ahí su capacidad espumante y emulsificante. Las proteínas del suero de leche también son utilizadas en microencapsulación por su baja viscosidad y alta capacidad emulsionante. Esta fracción contiene 4 proteínas mayoritarias: β-lactoglobulina (50%), α-lactoalbúmina (20%), albúmina del suero bovino (10%) e inmunoglobulina (10%). Pueden utilizarse como aislados o concentrados, solas o en combinación con hidratos de carbono o caseinatos. En relación con las técnicas de microencapsulación, existen numerosos métodos y, en el caso de los aceites y lípidos, destaca con mucha diferencia la atomización, la más económica y flexible, seguida por la liofilización, extrusión, cocristalización, coacervación e inclusión molecular (Gharsallaoui y col., 2007).

Hasta el momento, se han realizado muy escasos estudios sobre microencapsulación del CLA (Kim y col., 2000; Jiménez y col., 2004, 2006, 2008; Lee y col., 2009; Costa y col., 2015), en los cuales siempre se utiliza como sustrato la forma ácida y la atomización como método de secado, y las matrices incluyen ciclodextrinas (Kim y col., 2000), proteínas del suero de la leche, solas y en combinación con maltodextrinas y goma arábiga (Jiménez y col., 2004, 2006, 2008), agar y almidón de maíz (Lee y col., 2009) y proteínas de guisante en combinación con maltodextrina y carboximetilcelulosa (Costa y col., 2015). En la mayoría de estos estudios el objetivo es analizar las caracteísticas fisico-químicas de los microencapsulados obtenidos (morfología, solubilidad, temperatura de transición vítrea) con influencia en sus posibilidades de adición a alimentos, así como evaluar su aceptabilidad desde el punto de vista sensorial. En los casos en que se evalúa el proceso oxidativo se realiza mediante medidas de pérdida de sustrato, índice de peróxidos, hexanal o absorción de oxígeno. Los resultados obtenidos en términos de oxidación se refieren fundamentalmente a la distinta eficacia de las matrices. Por ejemplo, Kim y col. examinaron diferentes formas de ciclodextrinas entre las que destacan la forma α (Kim y col., 2000), y el grupo de Jiménez y colaboradores destacan en sus trabajos la protección conferida por las proteínas del suero de la leche (Jiménez y col., 2004, 2006, 2008).

2.- Objetivos.

El objetivo general de esta Tesis es abordar el estudio del proceso de oxidación en productos lácteos, con especial atención a los más susceptibles a la oxidación lipídica, esto es productos lácteos funcionales adicionados con ácidos grasos poliinsaturados que sin embargo han sido escasamente estudiados hasta el momento: i) Las fórmulas infantiles, de extraordinaria importancia porque suponen la única fuente de nutrientes de los lactantes y es esencial garantizar su calidad y seguridad; ii) Los productos lácteos funcionales con ácido linoleico conjugado, de más reciente comercialización, cuyo comportamiento oxidativo es prácticamente desconocido.

Para la consecución de este trabajo se han abordado los siguientes objetivos parciales:

1.- Determinación cuantitativa de los compuestos de oxidación presentes en una gran variedad de productos lácteos comercializados, tradicionales y funcionales: desarrollo y aplicación de métodos específicos de extracción de lípidos así como de métodos cuantitativos que permitan una medida global y objetiva del proceso oxidativo desde los primeros estadíos hasta fases de oxidación avanzada.

2.- Evaluación del efecto del tratamiento térmico de esterilización y de la adición de ácidos grasos poliinsaturados en sistemas modelo de fórmulas infantiles.

3.- Evaluación del efecto de las condiciones de almacenamiento en la formación de compuestos oxidados en fórmulas infantiles comercializadas.

4.- Identificación y cuantificación de los compuestos de oxidación formados, volátiles y no volátiles, y estudios de cinética de oxidación, en sistemas modelo y productos comercializados con ácido linoleico conjugado.

5.- Desarrollo de estrategias para aumentar la estabilidad oxidativa del ácido linoleico conjugado, mediante la adición de antioxidantes fenólicos, y mediante microencapsulación en matriz láctea.

Para llevar a cabo estos objetivos, el plan de trabajo ha incluido los siguientes ensayos:

1- Ensayos de conservación de productos lácteos convencionales y funcionales en condiciones de comercialización.

2.- Ensayos de oxidación en fórmulas infantiles modelo.

3.- Ensayos de oxidación de fórmulas infantiles a temperaturas ambiente y moderadas.

4.- Ensayos de oxidación a temperaturas ambiente y moderadas de los aceites ricos en CLA adicionados a productos lácteos.

5.- Ensayos de oxidación de aceites ricos en CLA adicionados con antioxidantes fenólicos y microencapsulados en matriz láctea.

3.- Resultados y Discusión.

Los resultados obtenidos en la Tesis han sido publicados:

3.1.- Oxidación lipídica en productos lácteos funcionales.

García-Martínez MC and Márquez-Ruiz G. Lipid oxidation in functional dairy products (2009) Current Nutrition and Food Science 5: 209-216.

3.2.- Presencia de compuestos de oxidación lipídica en productos lácteos funcionales comercializados.

García-Martínez MC, Fontecha J, Velasco J, Holgado F and Márquez-Ruiz G. Occurrence of lipid oxidation compounds in commercialized functional dairy products (2018) International Dairy Journal 86: 27-35.

3.3.- Efecto del tratamiento de esterilización clásica en fórmulas líquidas infantiles modelo con base láctea, de inicio y de continuación, sobre la oxidación lipídica.

García-Martínez MC, Holgado F, Velasco J and Márquez-Ruiz G. Effect of classic sterilization on lipid oxidation in model liquid milk-based infant and follow-on formulas (2012) European Journal of Lipid Science and Technology 114: 1373-1380.

3.4.- Efecto del tratamiento de esterilización en fórmulas líquidas infantiles embotelladas: impacto sobre la oxidación lipídica y el contenido de tocoferoles.

Márquez-Ruiz G, **García-Martínez MC** and Holgado F. Effect of sterilization of bottled infant milk: impact on lipid oxidation and tocopherols, in “Handbook of dietary and nutritional aspects of bottle feeding”, ed. VR Preedy, RR Watson and S Zibadi, Wageninen Academic Publishers, The Netherlands 2014, pp. 221-237.

3.5.- Estabilidad lipídica de fórmulas infantiles en polvo almacenadas a temperatura ambiente.

García-Martínez MC, Rodríguez-Alcalá LM, Marmesat S, Alonso L, Fontecha J and Márquez-Ruiz G. Lipid stability in powdered infant formula stored at ambient

temperatures (2010) International Journal of Food Science and Technology 45: 2337-2344.

3.6.- Compuestos de oxidación volátiles en un aceite rico en ácido linoleico conjugado.
García-Martínez MC, Márquez-Ruiz G, Fontecha J and Gordon MH. Volatile oxidation compounds in a conjugated linoleic acid-rich oil (2009) Food Chemistry 113: 926-931.

3.7.- Oxidación de un aceite funcional rico en CLA: determinación de compuestos volátiles y no volátiles.
Márquez-Ruiz G, Holgado F, Ruiz-Méndez MV, Velasco J and **García-Martínez MC**. Oxidation of a functional, CLA-rich oil: determination of volatile and non-volatile compounds (2016) European Food Research and Technology 242: 1993-2000.

3.8.- Efectividad del α-, γ- y δ-tocoferol en un aceite rico en CLA.
Márquez-Ruiz G, **García-Martínez MC**, Holgado F and Velasco J. Effectiveness of α-, γ- and δ-tocopherol in a CLA-rich oil (2014) Antioxidants 3: 176-188.

3.9.- La microencapsulación de aceites ricos en ácido linoleico conjugado (CLA) con componentes de leche desnatada protege frente a la polimerización.
Holgado F, **García-Martínez MC**, Velasco J, Ruiz-Méndez MV and Márquez-Ruiz G. Microencapsulation of conjugated linoleic acid (CLA)-rich oils with skimmed milk components protects against polymerization. Journal of the American Oil Chemists Society. DOI:10.1002/aocs.12146

3.1.- Oxidación lipídica en productos lácteos funcionales.

Lipid oxidation in functional dairy products.
María del Carmen García-Martínez y Gloria Márquez-Ruiz.
Current Nutrition and Food Science, 2009, 5: 209-216.

Resumen:
Generalmente los productos lácteos funcionales se enriquecen o fortifican con lípidos insaturados, conocidos por sus efectos beneficiosos para la salud, principalmente en la prevención de enfermedades cardiovasculares y de cáncer. Los lípidos mayoritariamente utilizados son ácidos grasos poliinsaturados de cadena larga omega-3, ácido linoleico conjugado o fitoesteroles. La oxidación lipídica puede ocurrir durante el procesamiento y almacenamiento de los productos lácteos funcionales enriquecidos con dichos lípidos insaturados y dar lugar a la aparición de sabores y olores desagradables así como a la formación de compuestos no saludables. Esta revisión subraya la importancia de analizar la estabilidad oxidativa de los productos lácteos funcionales para asegurar así la integridad de los lípidos originales y por consiguiente garantizar la calidad y seguridad de los productos lácteos funcionales. La revisión de los artículos científicos recientes muestra que existe escasa información sobre la oxidación lipídica en productos lácteos funcionales, debido en parte por constituir alimentos complejos. Los protocolos de la mayoría de estos estudios se basan en el enriquecimiento de la leche, total o parcialmente desnatada, o de los productos lácteos con lípidos funcionales, y examinan la oxidación durante el almacenamiento. En esta revisión se hace particular énfasis en discutir los métodos utilizados para evaluar la oxidación. Entre ellos, la evaluación sensorial es esencial para asegurar la aceptación del consumidor, mientras que el análisis cuantitativo de los productos de oxidación lipídica no volátiles y el de los antioxidantes, destacan como los métodos más adecuados para determinar el estado de oxidación lipídica de los productos lácteos funcionales.

Lipid Oxidation in Functional Dairy Products

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Abstract: Functional dairy products are usually fortified or enriched with unsaturated lipids of known positive health effects, mainly in prevention of cardiovascular diseases and cancer. The main lipids used are omega-3 long-chain polyunsaturated fatty acids, conjugated linoleic acid and phytosterols. Lipid oxidation can occur during processing and storage of functional dairy products enriched with such unsaturated lipids and results in formation of undesirable off-flavours and unhealthy compounds. This review highlights the importance of studying oxidative stability of functional dairy products in order to ensure the integrity of the original lipids and hence guarantee the quality and safety of functional dairy products. A survey of the recent literature shows that there is still scarce information on lipid oxidation in functional dairy products, in part due to its complexity in these multiphase food systems. Experimental protocols of most of the studies found start from enrichment of plain milk or dairy products with functional lipids, and examine oxidation along storage. Particular emphasis is placed in this review on discussion of the methods used for evaluation of oxidation. Among them, sensory evaluation is essential to ensure consumer acceptance, while quantitative analysis of non-volatile lipid oxidation compounds and antioxidants stand out as the most adequate methods to determine the lipid oxidation state in functional dairy products.

Keywords: Lipid oxidation, functional dairy products, omega-3 fatty acids, conjugated linoleic acid (CLA), phytosterols.

INTRODUCTION

Recent advances in the area of functional dairy products have resulted in the production of a variety of products enriched with lipids of known beneficial health effects [1-7]. Commercially available products include functional dairy products enriched with omega-3 long chain polyunsaturated fatty acids (ω-3 PUFAs), one of the fastest growing food product in the US and Europe. Also, conjugated linoleic acid (CLA) and phytosterols are among the lipids with reported beneficial effects which are incorporated nowadays in functional dairy products. Among dairy products, those which are normally considered the best vehicles for incorporation of oils with high concentration of PUFAs and CLA are milk and yoghourt, due to their healthy image, wide consumption and adequate physical properties [8, 9].

Traditionally, lipid oxidation of dairy products has not been a major deterioration source because of the high proportion of saturated fatty acids in milk fat. In contrast, lipid oxidation may be an important problem in functional dairy products enriched with polyunsaturated lipids since these are highly susceptible to oxidation. Lipid oxidation could occur during processing and storage of functional dairy products and result in formation of undesirable off-flavours and unhealthy compounds which come from the originally healthy lipids. Therefore, beneficial effects of the lipids incorporated could be invalidated. Furthermore, the compounds formed could lead to adverse physiological effects in cardiovascular and cancer processes [10], precisely the same targets for potential health benefits of ω-3 PUFAs and CLA.

The process of lipid oxidation leads to formation of a multitude of compounds of different molecular weight and polarity which make it difficult to evaluate the degree of oxidation and this situation becomes even more complicated in the case of dairy products enriched with functional lipids since additional factors are involved [11-14]. Such factors are essentially derived from the presence of the other food components, free radicals, metal ions and antioxidants; and from the physical properties of the food system, including solubility of oxygen and size and distribution of the oil droplets. Furthermore, it is difficult to deduce general conclusions due to the variety of oxidation conditions (oxygen availability, light/dark, temperature, time, presence of antioxidants/prooxidants, etc.) and analytical methods (substrate loss, peroxide value, thiobarbituric acid value, sensory evaluation, volatile oxidation products, etc.) applied to evaluate oxidation since each of the methods commonly used is only applicable to particular stages of the oxidative process, and the information provided depends on the type of method chosen [10, 14, 15]. This review focuses on the present state-of-the-art of lipid oxidation in functional dairy products, with particular emphasis on the methods used for evaluation of oxidation. Studies reporting results in this context are included in Table **1**, also specifying the type of samples analyzed and the analytical methods used.

LIPID OXIDATION IN ω-3 PUFAS-ENRICHED DAIRY PRODUCTS

The importance of dietary ω-3 PUFA in human health has been recognized through numerous epidemiological and intervention studies. They cannot be synthesized in the

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Table 1. Main Characteristics of Studies Focused on Lipid Oxidation of Functional Dairy Products

Functional Lipids		Dairy Substrate	Analysis of Oxidation	Authors & Year	Reference
ω-3 PUFAs	Cod liver and tuna oils	Commercial plain milk	Peroxide value Volatile oxidation products Sensory evaluation	Let *et al.* 2003	[27]
	Cod liver and tuna oils	Commercial plain milk	Peroxide value Anisidine value Volatile oxidation products Sensory evaluation	Let *et al.* 2004	[28]
	Commercial oil rich in ω-3 PUFAs	Simulated fermented milk	Loss of substrate	Luna *et al.* 2004	[33]
	Cod liver oil	Commercial plain milk	Peroxide value Volatile oxidation products Sensory evaluation	Let *et al.* 2005	[29]
	Cod liver oil	Commercial plain milk and yoghourt	Peroxide value Tocopherols Volatile oxidation products Sensory evaluation	Let *et al.* 2005	[24]
	Commercial oil rich in ω-3 PUFAs	Commercial ω-3 PUFAs-enriched milk and fermented milk	Oxidized triglycerides	García-Martínez *et al.* 2006	[36]
	Cod liver oil	Commercial plain milk	Peroxide value Tocopherols Volatile oxidation products Sensory evaluation	Let *et al.* 2007	[30]
	Cod liver oil	Commercial plain milk	Peroxide value Volatile oxidation products Sensory evaluation	Let *et al.* 2007	[31]
	Cod liver oil	Commercial plain milk	Peroxide value	Sorensen *et al.* 2007	[32]
	Cod liver oil	Simulated yoghourt	Peroxide value Tocopherols Volatile oxidation products Sensory evaluation	Nielsen *et al.* 2007	[8]
	Fish oil	Commercial plain yoghourt, cheese, cream and butter	Sensory evaluation	Kolanowski and Weiβbrodt 2007	[34]
	Cod oil	Commercial plain milk	Peroxide value Volatile oxidation products Sensory evaluation	Jiménez-Alvarez *et al.* 2008	[23]
	Linseed oil	Simulated milk	Volatile oxidation products	Giroux *et al.* 2008	[37]
	Linseed oil	Simulated milk	Thiobarbituric acid value	Giroux *et al.* 2008	[38]
CLA	Ruminant supplementation	CLA-enriched ice cream	Peroxide value	González *et al.* 2003	[63]

(Table 1) Contd….

Functional Lipids		Dairy Substrate	Analysis of Oxidation	Authors & Year	Reference
	Clarinol G-80 ®	Raw milk Simulated chocolate mixed-milk	Volatile oxidation products Sensory evaluation Loss of substrate	Campbell *et al.* 2003	[64]
	Ruminant supplementation	CLA-enriched milk, butter and cheese	Sensory evaluation	Avramis *et al.* 2003	[59]
	Ruminant supplementation	CLA-enriched milk	Sensory evaluation	Jones *et al.* 2005	[60]
	Ruminant supplementation	CLA-enriched milk	Loss of substrate	Lynch *et al.* 2005	[62]
	Tonalin TG-80 ®	Commercial CLA-enriched dairy products	Loss of substrate	Rodríguez-Alcalá and Fontecha 2007	[66]
	Ruminant supplementation	CLA-enriched butter	Gas chromatography-mass spectrometry with olfactrometry Sensory evaluation	Mallia *et al.* 2008	[61]
	Free fatty acids	Commercial milk powder and butter Simulated yoghourt	Sensory evaluation	Jiménez *et al.* 2008	[65]

Studies included in each group of functional lipids are listed in ascending order of publication year.

human body and show many positive effects on prevention of cardiovascular disease, including hypertension, inflammation, arrhythmia, hyperlipidemia. They are also claimed to exert cancer inhibition, anti-allergy effects and improvement in learning ability [3, 7, 16]. Main ω-3 PUFA are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). DHA acts as an important factor ensuring proper development and function of the brain and visual function [3, 17, 18]. EPA and DHA have been qualified heart-health claim for the Food and Drug Administration's 2004 and its intake is recommended by nutritional organizations, as Dietary Guidelines for Americans, American Heart Association, United Kingdom Department of Health and European Academy of Nutritional Sciences [19, 15].

The current intake of ω-3 PUFAs in the industrialized world is lower than recommended by nutritional organizations since consumption of fish and other sea animals has decreased considerably during the last years. Besides increasing fish consumption, alternative ways to ensure an optimal ω-3 PUFAs intake are dietary supplements and food enrichment with purified fish oils or fish oil powders obtained by microencapsulation [4-6, 11, 20]. Increased levels of ω-3 PUFAs in certain dairy products can be also efficiently achieved by the use of selected bacteria, in the case of fermented milks [9] or by including fish oils or marine algae in animal diets [21, 22].

In general, the methods most widely applied for evaluation of lipid oxidation in functional dairy products are sensory analysis, volatiles determination and peroxide value. The deterioration of sensory properties can sometimes be perceived readily and is a major cause for rejecting a product. The off-flavours formed from ω-3 PUFAs oxidation are particularly unpleasant and have a low odour threshold. Hence the presence of volatile secondary oxidation products, even at low concentrations, significantly decreases the sensory quality of fish oil and fish oil-containing foods. This is the reason why most of the studies examining oxidative stability of ω-3 PUFAs -enriched dairy products are exclusively focused in the determination of volatiles and sensory analysis.

Sensory evaluation is a specialized discipline, using trained panels to measure and analyze the characteristics of food lipids evoked by the sense of taste, smell, sight and mouth feel. However sensory analyses usefulness is limited because they are costly and require a well-trained taste and odour panels and the proper facilities. The main limitations often encountered in sensory techniques include poor reproducibility of the data and requirement of relative large amounts of samples. A better approach is to use precise chemical and instrumental methods to complement and support the sensory analyses.

Determination of volatile oxidation compounds show normally good concordance with peroxide value and sensory analysis in dairy products enriched with ω-3 PUFAs [23], although sometimes the method lacks sensitivity [24]. The main problem of exclusively determining volatile oxidation compounds is that the amount of oxidation compounds that hypothetically could be consumed is ignored.

Due to its simplicity, peroxide value is the analytical index normally used as control parameter of lipid oxidation in functional dairy products. It provides information on the content of primary oxidation compounds, i.e. hydroperoxides, formed at early oxidation stages. The determination

of peroxide value is very useful for bulk oils that can be analyzed directly. For foods, lipids need to be extracted with a mixture of solvents that must be carefully removed by evaporation without decomposition of hydroperoxides. Also, reducing substances co-extracted with the lipids by the polar solvents may lead to lower peroxide values [25]. In polyunsaturated oils, such as fish oils containing ω-3 PUFAs, the peroxide value maximum occurs at early stages of oxidation because of rapid and significant decomposition of hydroperoxides to yield secondary oxidation products, hence other complementary methods should be used to evaluate oxidation [20, 26].

As shown in Table **1**, the series of works published by Jacobsen and co-workers is particularly relevant in the context of lipid oxidation in dairy products enriched with ω-3 PUFAs [8, 11, 27-33]. The extensive works of these authors have in common the elaboration of ω-3 PUFAs-dairy products using commercial milk as substrate. The general procedure consisted in adding fish oil, often cod liver oil, to commercial milk and homogenizing in order to get milk emulsions containing 1.0% w/w milk fat and concentrations ranging between 0.25 to 1.5% w/w of fish oil. Starting fish oil quality in all studies was always good, showing very low peroxide values and acceptable concentration of tocopherols. The samples were usually stored at very low temperatures, except for one experiment carried out at 9ºC [24]. The methodology used combined the sensory analysis and volatiles determination with peroxide value and in some cases with antioxidant loss.

In general, the results obtained by Jacobsen and coworkers under the conditions selected showed very low oxidation values. Among the relevant results obtained by the authors, it is interesting to comment some general observations. Even though peroxide value in milk emulsions containing fish oil increased significantly during storage, final levels were always low [24, 28, 29]. Based on peroxide values and volatiles, oxidation was not detected in dairy products enriched with fish oil throughout storage, except for samples in which the oil antioxidants have been removed or the homogenization conditions were more unfavourable [29, 32]. Milk enriched with cod liver oil had a distinct fishy off-flavour already after a 1 day-storage [27], nevertheless, the development of rancid or metallic off-flavours during the storage of samples was low [24, 30, 27]. Yoghourt was the subtract that suffered less sensory alteration after addition of fish oil, there was no development in aroma or flavour over time except for a very small increase in rancid flavour after 8 or 14 days of storage [30]. Similarly, other authors have found that fermented milks enriched with ω-3 PUFAs did not undergo changes in fatty acid composition or volatile profile typical of yoghourts and fermented milks [33]. With respect to the effect of homogenization conditions and protein composition, results indicated that composition of the oil-water interface was more significant to oxidation than total surface area, i.e., droplet size [31] and that a combination of more β-lactoglobulin and less casein at the oil-water interface provided greater oxidative stability [32].

One of the most important conclusions of the authors was that sensory analysis was more sensible than peroxide value and volatiles determination since the trained panels detected fishy off-flavours at low concentrations of volatiles or peroxide value [8]. Also it was found that volatile secondary oxidation products normally had a good correlation with peroxide value and that anisidine values had no correlation with the sensory characteristics and was not a sufficiently sensitive analysis [24]. Based on sensory evaluation, Kolanowski and Weibbrodt evaluated the influence of fish oil fortification on the sensory quality of yoghurts, fresh, soft and processed cheeses, butter and cream. They concluded that the level of fortification of dairy products with ω-3 PUFAs was limited, being the highest acceptable level for solid, high-fat dairy products (spreadable fresh cheese, butter and processed cheeses), especially when flavourings were present [34].

A quite different approach to evaluate oxidation in ω-3 PUFAs-enriched dairy products is to quantitate major non-volatile oxidation compounds, that is, oxidized triglycerides. Relevant information on the state and evolution of oxidation has been obtained when analyzing oxidized triglycerides in microencapsulated fish oils, often used as functional ingredientes in dairy products [20, 26, 35]. Examination of oxidized triglycerides levels in commercial ω-3 PUFAs-dairy products showed significant increases of oxidized triglycerides at the expiry date [36].

Addition of antioxidants and chelating agents is proposed to protect foods enriched in ω-3 PUFAs but their efficacy in such complex food systems is difficult to predict [11]. Regarding alternatives to increase protection against oxidation, Giroux and coworkers have recently reported the advantages of using innert gases [37] and have also proposed to apply electroreduction [38], since good results were obtained when assayed in dairy beverages supplemented with linseed oil.

Additional studies not focused on lipid oxidation but examining the organoleptic effects of adding ω-3 PUFAs to dairy products, especifically fermented milks, have reported no effect on yoghurt flavour whereas texture was adversely affected [39]. However, other experiments have shown that fortification of yoghourts with polyunsaturated oils did not have any effect on microbial growth whereas texture and flavour were both adversely affected [40, 41].

Even though the studies carried out so far show generally good oxidative stability for the ω-3 PUFAs-enriched dairy products tested, it should be kept in mind that practically all samples analyzed had been added with ω-3 PUFAs during the experimental design, that is, they were not commercial dairy products enriched with ω-3 PUFAs. Hence it should not be disregarded that significant oxidation may occur under different conditions applied in the dairy industry, during processing and storage, such as temperature and time for homogenization and sterilization, and storage temperature.

LIPID OXIDATION IN CLA-ENRICHED DAIRY PRODUCTS

Conjugated linoleic acid (CLA) is a collective term embracing all octadecadienoic acids (C18:2) isomers with conjugated double bonds. Antioxidant and anti-cancer properties have been attributed to CLA, and studies on mice

and rats show encouraging results in hindering the growth of tumors in mammary, skin, and colon tissues [42-47]. Additionally, CLA has shown effects on reduction of body fat and enhancement of muscle mass [48-50]. A valuable source of information concerning research on CLA can be found in the webpage maintained by Pariza (http://fri.wisc.edu/clarefs.htm).

Formation of CLA occurs naturally in ruminants and hence dairy products contain CLA quantities ranging from 5 to 30 mg/g fat [51, 52]. Estimates of CLA daily intake from food sources range from 212 mg/day to 1.5 g/day [53, 54]. However, it has been estimated that a 70-kg human should consume 3.0 g of CLA/day to achieve maximum health benefits [44]. There are different approaches to increasing CLA contents in milk products. Modifying ruminants diets with supplements rich in polyunsaturated fatty acids is rather effective [55, 56] although increased levels are obtained by directly adding CLA-rich oils to dairy products. Most of such CLA-rich oils are obtained by processes that convert linoleic acid from linoleic acid-rich vegetable oils, usually safflower or sunflower oils, into mainly C18:2 *cis*-9, *trans*-11 and C18:2 *cis*-10, *trans*-12 isomers, approximately in similar proportions, plus other minor CLA isomers [1]. Increased levels of CLA in the case of fermented milks can be also efficiently achieved by the use of selected bacteria [57].

Studies on the shelf-life stability of CLA-enriched dairy products generally show no significant differences in flavour quality parameters [58]. Several reports on dairy products enriched through ruminant dietary supplements have been found and all agree in that nule or very small effects were found due to CLA fortification. Milk enriched in CLA by a fish meal supplemented diet did not lead to increased oxidized flavour as compared with the control milk nor had influence on flavour of CLA-enriched cheeses [59, 60]. Likewise, CLA-rich butter prepared with milk from cows fed sunflower seeds did not show different aroma profiles by olfactometric analysis in initial samples, and no significant differences were detected in attributes related to oxidation as compared to control butter after 6 weeks of storage [61]. Lynch and coworkers exposed pasteurized milk rich in CLA to light over 14 days and did not find effect on fatty acid composition [62]. González and coworkers investigated the shelf-life in ice cream made out of milk containing high CLA levels and found even lower peroxide values for enriched ice cream as compared to control ice cream [63].

In relation to products added with CLA-rich oils, wherein the content of CLA is much higher, Campbell and coworkers analyzed the impact of highly CLA-fortified dairy beverage (CLA content up to 81.9% of fatty acid composition) and did not find significant differences in hexanal or other common indicators of lipid oxidation between milks with or without added CLA [64]. Sensory evaluation of dairy products supplemented with microencapsulated CLA has shown that the threshold was higher in butter (300 mg per 100 g) followed by yoghourt and finally milk, indicating that CLA was much harder to detect in butter [65]. Changes in fatty acid composition of 6 commercially available CLA-fortified dairy products were analyzed during processing and 10 weeks after refrigeration. Dairy products were based on skim milk fortified with CLA-rich oil and included milk, milk powder, fermented milk, yogurt, fresh cheese and milk-juice blend. All dairy products contained 1.2 g of CLA (about 80% were *cis*-9, *trans*-11 and *cis*-10, *trans*-12 isomers in similar proportions). Refrigerated storage and thermal processing resulted in significant decreases or disappearance of some of the minor CLA isomers while a slight but significant increase of *trans, trans* isomers was observed especially in CLA-fortified milk powder [66]. *Trans, trans* isomers are found as minor compounds either in natural CLA of dairy products [67] or in synthetic CLA mixtures [68], their increases have been associated with heat treatments [69] but little is known about their possible metabolic effects [70].

Evaluation of CLA oxidation, as well as ω-3 PUFAs oxidation, is often determined by substrate loss, through calculating its decrease in total composition of fatty acid methyl esters derivatives analyzed by gas chromatography [33, 62, 66]. Fatty acid composition is routinely used by manufacturers to ensure the level of functional fatty acids added to dairy products. However, it has shown to be less sensitive and less accurate than direct determination of the oxidation compounds formed, since significantly high oxidation may occur prior to detection of substrate loss [71, 72].

Measurement of CLA oxidation is controversial since it is generally agreed that oxidation pathways of CLA are unclear [72-77] and it appears that, in contrast to the case of linoleate, hydroperoxides are only minor products in the oxidation of conjugated linoleate. Therefore, the methods normally used to control lipid oxidation in foods, e.g., peroxide value and thiobarbituric acid reactive substances, may not indicate the real level of oxidation and provide misleading results. Otherwise, it has been reported that formation of polymers occurs from the very beginning of the oxidation process [72]. Accordingly, volatile profile found in oxidized CLA-rich oil was not that expected from theoretical hydroperoxides formed from CLA, showing significantly high quantities of heptanal, *t*-2-octenal and *t*-2-nonenal [77]. Therefore, hexanal content is not an useful marker of the oxidation progress in CLA-rich oils and, in general, differences in volatile profile are likely to be reflected in sensory evaluations and should be further investigated.

LIPID OXIDATION IN PHYTOSTEROLS-ENRICHED DAIRY PRODUCTS

Phytosterols are plant derived sterols that have similar structure as cholesterol thus interfere with the uptake of cholesterol from the intestinal tract and consequently lower the cholesterol level in the bloodstream [78]. Jones *et al.* [79] showed the effectiveness of the inclusion of 1.7 g phytosterols/day into the diet of hypercholesterolemic men. The use of phytosterols in dairy products is quite extended and their benefits well demonstrated [80-84]. Plant stanols are a saturated subgroup of sterols and are also used as potent hypocholesterolemic agents in dairy products [85].

Even though phytosterol oxidation products can trigger cytotoxic effects comparable to those associated with cholesterol oxidation products, there are few studies on the formation and analysis of phytosterol oxidation products

[86]. A major challenge for analysis of phytosterol oxidation products is that numerous oxidation compounds can be formed and analysis becomes even more complicated in dairy products that contain also cholesterol oxidation products. The situation is worsened by the fact that reference compounds for phytosterol oxidation products are not available commercially [87].

To our knowledge, there is no information published so far on the analysis of phytosterol oxidation in functional dairy products, which would be of great interest particularly when unsaturated phytosterols are used and if this is done in combination with ω-3 PUFAs.

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3.2.- Presencia de compuestos de oxidación lipídica en productos lácteos funcionales comercializados.

Occurrence of lipid oxidation compounds in commercialized functional dairy products.

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Resumen:

La estabilidad oxidativa de los lípidos funcionales es esencial para asegurar su acción bioactiva en la prevención o tratamiento de determinadas enfermedadas.

En este estudio el estado de oxidación de productos lácteos funcionales comerciales, junto con productos lácteos convencionales, se evaluó en las fechas de adquisición y caducidad. Se analizaron productos lácteos funcionales que contienen ácidos grasos omega-3 (FPω3), ácido linoleico conjugado (FPCLA) o ésteres de estanol (FPSE). La cuantificación de triacilgliceroles oxidados mediante extracción en fase sólida y cromatografía de exclusión por tamaño de alta resolución, metodología no aplicada hasta ahora a los productos lácteos, se utilizó en la determinación de los compuestos de oxidación primarios y secundarios. Los tocoferoles y el índice de peróxidos también fueron analizados. Los productos lácteos convencionales y FPSE mostraron una elevada estabilidad, en contraste con la estabilidad relativamente inferior de FPω3 y FPCLA. Los productos lácteos funcionales con omega-3 y ácido linoleico conjugado contenían compuestos de oxidación incluso en la fecha inicial de adquisición, y la mitad de dichas muestras se oxidaron aún más durante el período de vida útil. Sin embargo, mientras que los hidroperóxidos fueron los compuestos predominantes en productos lácteos funcionales con omega-3, se descubrió que la formación de polímeros era el marcador de oxidación temprana en la oxidación de productos lácteos funcionales con ácido linoleico conjugado.

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Occurrence of lipid oxidation compounds in commercialised functional dairy products

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ABSTRACT

Oxidative stability of bioactive lipid-functional dairy products is essential to ensure their role in the prevention and/or treatment of certain diseases. The oxidation state of commercial functional dairy products, along with conventional dairy products, was evaluated at the acquisition and expiry dates. Functional dairy products containing omega-3 fatty acids (FPω3), conjugated linoleic acid (FPCLA) or stanol esters (FPSE) were tested. Quantitation of oxidised triacylglycerols, not applied so far to dairy products, was used as a measurement of primary and secondary oxidation compounds. Tocopherols and peroxide values were also determined. Conventional dairy products and FPSE showed high stability, in contrast to the relatively lower stability of FPω3 and FPCLA. FPω3 and FPCLA were significantly oxidised even at the initial date, and half of such samples oxidised further during the shelf-life period. However, while hydroperoxides were predominant in FPω3, polymer formation was found to be the early oxidation marker of FPCLA oxidation.

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1. Introduction

Lipid oxidation in foods is of great concern because it results in development of undesirable flavours, decreased shelf-life, loss of nutritional value and formation of compounds that may be detrimental to health (Dobarganes & Márquez-Ruiz, 2003; Kanner, 2007). During the last decade, consumers' demands for dairy-based functional foods have increased considerably; enrichment with functional lipids, including omega-3 polyunsaturated fatty acids (ω-3 PUFAs), conjugated linoleic acid (CLA) or plant sterols make up a significant part of such foods (Gulseven & Wohlgenant, 2014).

A large body of scientific research has confirmed the health-promoting properties of ω-3 PUFAs concerning risk reduction of cardiovascular diseases (Shahidi, 2015), while CLA isomers are mainly claimed to prevent tumours growth or to reduce body fat (Yang et al., 2015). Plant sterols, in turn, have consistently shown total and low-density lipoprotein cholesterol-lowering effects (Gylling et al., 2014).

Lipid oxidation is not the main deterioration source in dairy products because of the high proportion of stable, saturated fatty acids of milk fat, although oxidation reactions may be promoted by heating treatments with oxygen exposure during processing and storage (Duncan & Webster, 2010; Fenaille et al., 2006; García-Martínez et al., 2010; Márquez-Ruiz, García-Martínez, & Holgado, 2014; Smet et al., 2009; Stapelfeldt, Nielsen, & Skibsted, 1997). However, ω-3 PUFAs and CLA are polyunsaturated fatty acids, therefore highly susceptible to form oxidised compounds with adverse physiological effects (Dobarganes & Márquez-Ruiz, 2003; García-Martinez & Márquez-Ruiz, 2009).

Most studies on the oxidative stability of functional dairy products have focused on those enriched with ω-3 PUFAs, as reported by Jacobsen, 2010; Let, Jacobsen, Frankel, & Meyer, 2003, 2004, 2005, 2007; Saga et al., 2013). Interest in oxidative stability of functional dairy products enriched with CLA or sterol plants is also increasing, as reflected in recent publications (González-Larena, Garcia-Llatas, Clemente, Barberá, & Lagarda, 2015; Martínez-Monteagudo, Leal-Dávila, Curtis, & Saldaña, 2015; Nieminen, Leaksop, Kuusisto, Niemela, & Laitinen, 2016). However, very little is known about the state of oxidation of commercially available functional dairy products. To the best of our knowledge, the only information published in this regard refers to fatty acid profiles of commercial CLA-fortified dairy products (Rodríguez-Alcalá &

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Fontecha, 2007) and to sterol oxides in three commercial plant sterol-enriched dairy products (García-Llatas et al., 2013; González-Larena et al., 2015).

Different methods have been used to evaluate lipid oxidation in dairy products, such as peroxide value, loss of substrate, thiobarbituric acid reactive substances, volatile oxidation compounds, loss of antioxidants and sensory analysis (García-Martinez & Márquez-Ruiz, 2009). However, each of these methods only detects compounds formed during a particular stage of the oxidation process (Barriuso, Astiasaran, & Ansorena, 2013; Dobarganes & Márquez-Ruiz, 2003; Frankel & Meyer, 2000). Even when some of these methods are used in combination, the real level of oxidation compounds formed remains unknown.

The application of combined chromatographic techniques can provide a complete picture of the oxidation state at any stage of the oxidation progress (Dobarganes, Velasco, & Dieffenbacher, 2000; Márquez-Ruiz, Jorge, Martín-Polvillo, & Dobarganes, 1996). This method has provided excellent results in many food samples, including vegetable oils, fish oils, fried foods, microencapsulated oils and infant formulas (Dobarganes & Márquez-Ruiz, 2013; García-Martínez et al., 2010; Giménez, Gómez-Guillén, Pérez-Mateos, Montero, & Márquez-Ruiz, 2011; Márquez-Ruiz & Dobarganes, 2006; Márquez-Ruiz, Velasco, & Dobarganes, 2000), but it has not yet been applied to conventional or functional dairy products.

The aim of the present work was to provide new information on the effect of addition of functional lipids on the oxidative stability of dairy products. Towards this end, the oxidation state in a variety of commercial functional dairy products, along with conventional dairy products, was evaluated at initial and expiry dates through quantitation of non-volatile oxidation compounds by combined chromatographic techniques. Also, fatty acid composition, tocopherol contents and peroxide values were determined.

2. Materials and methods

2.1. Samples and reagents

In this benchmarking study, 12 functional dairy products and 10 conventional dairy products from the most representative national and international manufacturers were acquired in local supermarkets. A brief description of each product and total fat content, as stated in the labels, along with the abbreviations used in this study, is listed in Table 1. Also, the shelf-life, according to the expiry date, was included for all products. The functional dairy products selected included 6 products enriched with ω-3 PUFAs (FPω3), 4 products with added CLA (FPCLA), and 2 products enriched with stanol esters (FPSE). As regards conventional dairy products, 2 pasteurised whole milks (PM), 2 ultra-high temperature-treated whole milks (UHTM), 2 fermented milks (FM), 2 evaporated whole milks (EM) and 2 powdered whole milks (POM) were selected.

Six containers of the same lot were acquired for each commercial product; 3 of these were immediately analysed in triplicate, the other 3 were stored in their original containers (sealed) until the expiry date stated by the manufacturer (the duration of storage is included in Table 1) and then analysed. Products were stored at their respective recommended temperatures, i.e., refrigeration temperature (2–4 °C) for PM-A, PM-B, FM-A, FM-B, FPω3-E, FPCLA-C, FPCLA-D, FPSE-A and FPSE-B; and room temperature (20–23 °C) for the rest of the samples.

All reagents used for lipid extraction and separation of polar compounds by SPE were purchased from Panreac (Barcelona, Spain). An anhydrous milk fat (reference material BCR-164; EU Commission, purchased from Fedelco Inc., Madrid, Spain) was used for quantitative determinations of total fatty acid methyl esters. Methyl esters of ω-3 fatty acids and CLA were identified by gas chromatography-flame ionisation detection (GC-FID) using standards purchased from Nu-Check Prep. Inc. (Elysian, MN, USA). The chemicals and solvents used for other methods were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Lipid extraction

Lipids were extracted according to the International Standard Method ISO 14156-IDF for milk and milk products (ISO-IDF, 2002), except for POM samples where two lipid fractions were extracted separately, i.e., the free, non-encapsulated and the encapsulated fat fractions. The extraction of the free fat was carried out according to Sankarikutty, Sreekumar, Narayanan, and Mathew (1988). Thus,

Table 1
Brief description of the conventional and functional dairy products analysed.

Commercial product	Description (as in label)	Total fat (g 100 mL^{-1}; as in label)	Shelf-life (days)	Brand	Abbreviation
Pasteurised milk	Whole milk	3.7	10	A	PM-A
	Whole milk	4.4	12	B	PM-B
Ultra high temperature milk	Whole milk	4.2	90	A	UHTM-A
	Whole milk	3.8	94	B	UHTM-B
Fermented milk	Whole milk	4.0	23	A	FM-A
	Whole milk	3.8	19	B	FM-B
Evaporated milk	Whole milk	19.0	240	A	EM-A
	Whole milk	18.2	216	B	EM-B
Powdered milk	Whole milk	30.0	498	A	POM-A
	Whole milk	34.5	463	B	POM-B
Functional dairy products with ω-3 polyunsaturated fatty acids	Skimmed milk (SM) with vegetable oils and fish oil	2.8	65	A	FPω3-A
	SM with high-oleic sunflower, olive and fish oils	2.9	54	B	FPω3-B
	SM with high-oleic sunflower, olive and fish oils	3.0	62	C	FPω3-C
	SM with virgin olive oil and marine oils	3.3	75	D	FPω3-D
	Fermented SM (FSM) with high-oleic sunflower, olive and fish oils	2.0	18	E	FPω3-E
	SM with sunflower, corn and fish oils	1.6	71	F	FPω3-F
Functional dairy products with conjugated linoleic acid (CLA)	SM with oil rich in CLA	1.0	79	A	FPCLA-A
	SM with oil rich in CLA	1.5	87	B	FPCLA-B
	FSM with oil rich in CLA	2.0	18	C	FPCLA-C
	FSM with oil rich in CLA	1.5	19	D	FPCLA-D
Functional dairy products with plant sterols	FSM with stanol esters	5.0	20	A	FPSE-A
	FSM with stanol esters	4.6	22	B	FPSE-B

200 mL of light petroleum ether (60–80 °C) was added to 4 g of powder. Stirring was applied at room temperature for 15 min. After filtration through a filter paper, the solvent was evaporated in a rotatory evaporator and the extracted oil was dried to constant weight using a stream of nitrogen. The extraction of encapsulated fat was carried out in the powder sample devoid of free fat and dried to constant weight, following the International Standard Method ISO 14156-IDF (ISO-IDF, 2002). Extracted lipids for all dairy samples were calculated as weight percentage (grams of lipids extracted from 100 g of product).

2.3. Peroxide value

Peroxide value (PV) was determined in extracted lipids by the iodometric assay according to IUPAC Standard Method 2.501 (IUPAC, 1992).

2.4. Fatty acid composition

Extracted lipids were converted into fatty acid methyl esters (FAMEs) by base-catalysed transmethylation using 2 N KOH in methanol, as described by International Standard Method ISO 15884-IDF (ISO-IDF, 2002). FAMEs were analysed on a Perkin–Elmer chromatograph (model Autosystem, Beaconsfield, UK) with FID. FAMEs were separated using a fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness, CP-Sil 88, Chrompack, Middelburg, the Netherlands). The column was held at 100 °C for 1 min after injection, then the temperature was increased at 7 °C min^{-1} to 170 °C, held for 55 min, then increased at 10 °C min^{-1} to 230 °C, and held there for 33 min. Helium was the carrier gas with a column inlet pressure set at 214 kPa (30 Psig) and a split ratio of 1:20. The injection volume was 0.5 μL.

2.5. Non-volatile oxidation compounds

Quantitative analysis of total non-volatile oxidation compounds in extracted lipids was carried out according to the method described by Márquez-Ruiz and Dobarganes (2006) and Márquez-Ruiz et al. (1996). Oxidation compounds were separated from the non-oxidised triacylglycerols by solid-phase extraction (SPE). Briefly, 50 mg of extracted lipids were dissolved in 2 mL of hexane containing 1 mg of monostearin added as internal standard. The sample solution was poured into a 1 g silica Sep-Pak cartridge (Waters, Darmstadt, Germany) and the non-oxidised triacylglycerols were eluted with 15 mL hexane/diethyl ether (90:10, v/v). After that, the polar fraction was eluted with 25 mL diethyl ether. The solvent of the polar fraction was evaporated in a rotary evaporator and redissolved in 1 mL diethyl ether. The polar fraction was analysed by high performance size exclusion chromatography in a liquid chromatograph equipped with a Rheodyne injector with a 20 μL sample loop, a Waters 510 pump (Waters, Milford, MA, USA), and a Waters refractive index detector. The separation was performed on two PLgel columns (Agilent Technologies, Palo Alto, CA, USA) packed with 5 μm particles of 100 and 500 Å pore size, respectively, and placed into an oven set at 35 °C. High performance liquid chromatography (HPLC) grade tetrahydrofuran was the mobile phase with a flow of 1 mL min^{-1}. Under these conditions, resolved peaks of triacylglycerol polymers (TGP), triacylglycerol dimers (TGD), oxidised triacylglycerol monomers (oxTGM), diacylglycerols (DGs), monostearin (internal standard), and finally a peak corresponding to free fatty acids of various chain lengths and the polar unsaponifiable matter, were obtained. Total non-volatile oxidation compounds were calculated as the sum of TGP, TGD and oxTGM. This methodology was described in detail, including precision and repeatability data by Márquez-Ruiz et al. (1996).

2.6. Tocopherols

Determination of tocopherols in extracted lipids was carried out by HPLC with fluorescence detection according to IUPAC Standard Method 2.411 (IUPAC, 1992).

2.7. Statistical analysis

Data were analysed using the ANOVA procedure of the SPSS package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL). Tukey's test was applied to determine significance and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Fatty acid composition and lipid content of initial dairy products

Tables 2 and 3 show the composition of the fatty acid methyl esters (% of FAMEs) and lipid content of all the dairy products analysed. The lipid contents of PM, UHTM and FM samples were within the levels expected, between 3.6 and 4.2% (grams of lipids in 100 g of product), and were also, as expected, higher in the evaporated and dried milks, consistent with the dehydration degree applied. Very similar fatty acid compositions were found for the conventional milk products, matching with most common values for bovine milk fat (Rodríguez-Alcalá, Alonso, & Fontecha, 2014). The distinct fatty acid compositions found in the functional products derived from the contribution of different sources of oils or lipids added. All the FPω3 products had been enriched with fish oils, but in different amounts, as reflected by the ω-3 contents, ranging from 0.6 to 4.3%. The high oleic acid content in FPω3-A, FPω3-B, FPω3-D and FPω3-E accounted for the addition of olive oil and/or high-oleic sunflower oil, as stated on the labels (Table 1). In contrast, the major fatty acid in FPω3-F was linoleic acid, highly abundant in sunflower oil and other seed oils commonly used in the preparation of functional products. As regards FPω3-C, the significant proportions of palmitic acid, short-chain and medium-chain fatty acids found indicate that the skimmed milk used contained some remaining milk fat. The same was observed in some functional dairy products enriched with CLA oil, especially in FPCLA-A, as already reported (Rodríguez-Alcalá & Fontecha, 2007). In this line of functional products, similar concentrations of *cis*-9, *trans*-11 CLA and *cis*-10, *trans*-12 CLA were found, in sum ranging from 52.2 to 75.8%. The functional dairy products with plant sterols were abundant in monounsaturated fatty acids coming from the vegetable oils used as plant stanol vehicles (García-Llatas et al., 2013).

3.2. Oxidation parameters in conventional dairy products

Table 4 shows values of PV, quantitative data of non-volatile, triacylglycerol oxidation compounds (oxTGM, TGD and TGP) and tocopherol contents in initial conventional dairy products. All the conventional dairy products showed PV below 1 meq O_2 kg^{-1} oil and oxidation compounds as determined by chromatographic techniques were not detectable, except for the POM samples. In POM-A and POM-B, 9.6% and 9.9% of the total lipids was non-encapsulated or free, respectively, and results showed that he free lipid fractions were significantly more oxidised than the

Table 2
Fatty acid composition and total lipids extracted from initial conventional dairy products.[a]

FAME (wt% on oil)	PM-A	PM-B	UHTM-A	UHTM-B	FM-A	FM-B	EM-A	EM-B	POM-A	POM-B
C4:0	3.3 ± 0.32	3.4 ± 0.20	3.2 ± 0.17	3.1 ± 0.05	3.2 ± 0.27	3.1 ± 0.58	3.5 ± 0.26	3.0 ± 0.20	3.8 ± 0.18	3.6 ± 0.26
C6:0	2.6 ± 0.21	2.4 ± 0.03	2.5 ± 0.13	2.4 ± 0.22	2.2 ± 0.11	2.1 ± 0.09	2.4 ± 0.16	2.3 ± 0.17	2.4 ± 0.12	2.3 ± 0.12
C8:0	1.1 ± 0.08	1.0 ± 0.05	1.1 ± 0.06	1.0 ± 0.01	1.1 ± 0.04	1.2 ± 0.06	1.1 ± 0.07	1.2 ± 0.06	1.3 ± 0.07	1.3 ± 0.06
C10:0	2.6 ± 0.12	2.6 ± 0.04	2.5 ± 0.06	2.3 ± 0.22	2.5 ± 0.10	2.6 ± 0.05	2.9 ± 0.14	2.7 ± 0.08	2.9 ± 0.05	2.8 ± 0.11
C10:1	0.3 ± 0.02	0.2 ± 0.00	0.3 ± 0.01	0.3 ± 0.00	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.01	0.2 ± 0.01
C12:0	3.1 ± 0.07	3.1 ± 0.16	3.2 ± 0.05	2.9 ± 0.13	3.1 ± 0.07	2.9 ± 0.14	3.8 ± 0.06	2.8 ± 0.10	3.8 ± 0.09	3.5 ± 0.15
C13:0	0.2 ± 0.02	0.3 ± 0.02	0.2 ± 0.01	0.2 ± 0.04	0.3 ± 0.04	0.2 ± 0.01	0.3 ± 0.05	0.2 ± 0.04	0.3 ± 0.06	0.2 ± 0.03
C14:0	11.2 ± 0.33	10.9 ± 0.12	10.4 ± 0.65	11.3 ± 0.24	10.0 ± 0.49	10.0 ± 0.56	11.6 ± 0.46	11.8 ± 0.55	10.4 ± 0.70	10.4 ± 0.94
C15:0	1.0 ± 0.05	0.9 ± 0.03	1.1 ± 0.02	1.0 ± 0.05	0.9 ± 0.05	0.9 ± 0.02	0.9 ± 0.02	1.0 ± 0.08	0.9 ± 0.02	0.8 ± 0.06
C16:0	32.7 ± 0.87	34.7 ± 1.08	31.8 ± 1.10	34.3 ± 1.22	32.7 ± 1.29	31.6 ± 1.21	35.3 ± 1.38	33.5 ± 1.37	27.3 ± 1.30	29.0 ± 1.47
C16:1	2.0 ± 0.16	1.1 ± 0.02	0.2 ± 0.01	1.1 ± 0.03	1.2 ± 0.02	1.4 ± 0.02	2.0 ± 0.00	1.6 ± 0.02	1.5 ± 0.02	1.5 ± 0.01
C18:0	11.8 ± 0.22	12.3 ± 0.12	12.4 ± 0.60	11.9 ± 0.79	11.9 ± 0.50	11.9 ± 0.47	10.8 ± 0.72	12.0 ± 0.84	12.9 ± 0.75	12.5 ± 0.60
C17:0	2.0 ± 0.16	2.1 ± 0.07	1.9 ± 0.06	2.0 ± 0.03	2.0 ± 0.00	2.0 ± 0.02	2.1 ± 0.05	2.0 ± 0.08	1.9 ± 0.11	2.1 ± 0.05
C17:1	0.1 ± 0.02	0.1 ± 0.00	0.2 ± 0.01	0.1 ± 0.01	0.2 ± 0.02	0.2 ± 0.01	0.1 ± 0.01	0.1 ± 0.02	ND	ND
C18:1	23.6 ± 0.77	22.2 ± 0.34	22.7 ± 0.32	23.0 ± 1.25	23.0 ± 0.84	24.3 ± 1.21	21.1 ± 0.58	22.7 ± 0.67	25.9 ± 1.27	25.4 ± 0.90
C18:2 9*c*,12*c*	1.7 ± 0.16	1.1 ± 0.03	2.3 ± 0.01	1.7 ± 0.12	1.9 ± 0.02	1.9 ± 0.00	1.1 ± 0.02	1.0 ± 0.00	2.2 ± 0.01	2.2 ± 0.02
C18:2 9*c*,11*t*	0.1 ± 0.00	0.1 ± 0.01	0.1 ± 0.02	0.3 ± 0.03	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	ND	1.0 ± 0.00	0.7 ± 0.00
C18:2 10*t*,12*c*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:3 *n*-3	ND	0.2 ± 0.02	0.1 ± 0.03	0.2 ± 0.00	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.03	0.1 ± 0.01
C20:0	0.1 ± 0.02	ND	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.00	0.1 ± 0.00	ND	ND	0.1 ± 0.01	0.1 ± 0.02
C20:5 *n*-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C22:6 *n*-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SFA	71.7	73.7	70.4	72.5	69.0	67.6	74.7	72.5	68.0	68.6
∑MUFA	26.0	23.6	23.4	24.5	24.7	26.2	23.5	24.7	27.7	27.1
∑PUFA	1.8	1.4	2.5	2.2	2.2	2.2	1.3	1.1	3.3	3.0
Extracted lipids (wt% on product)	3.6 ± 0.32	4.2 ± 0.25	4.1 ± 0.28	3.7 ± 0.29	3.9 ± 0.20	3.7 ± 0.35	18.3 ± 2.51	17.1 ± 1.90	28.3 ± 2.08	34.8 ± 2.51

[a] Abbreviations are: FAME, fatty acid methyl esters; PM, pasteurised whole milk; UHTM, ultra-high temperature-treated whole milk; FM, fermented milk; EM, evaporated whole milk; POM, powdered whole milk; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected. Data are means ± SD ($n = 3$).

encapsulated fractions. Thus, 16.1 and 10.4 mg g^{-1} total oxidation compounds were respectively found in the free lipid fractions, in contrast to values of 1.5 and 2.3 mg g^{-1} observed in the encapsulated lipid fractions of POM-A and POM-B, respectively. Clearly, the free lipid fraction was more susceptible to oxidation than the fraction embedded in the matrix, as already reported in powder milks (Vignolles, Jeantet, Lopez, & Schuck, 2007) and powdered infant formulas (García-Martínez et al., 2010).

Following the shelf-life period, the samples were examined at their corresponding expiry dates. Since none of the conventional dairy products showed significant changes in any of the oxidation parameters measured, data has not been included. Even in the POM samples, which have been reported to oxidise significantly under certain storage temperature and water activity (Stapeldefeldt et al., 1997), no significant changes were found at the expiry dates in the present study, probably because of the protection provided by the vacuum-sealed bags used for packaging.

3.3. Oxidation parameters in functional dairy products

As compared with the negligible level of oxidation found in the conventional products (Table 4), the oxidation state in the initial time was apparently high in the functional products (Table 5). However, the results found were in accord with those reported for the refined vegetable oils used in their formulation. Thus, PV was always lower than the limit allowed for refined vegetable oils, 10 meq O_2 kg^{-1} oil, and a small amount of TGD remained as marker of the oil refining process (Ruiz-Méndez, Márquez-Ruiz, & Dobarganes, 1997).

The tocopherol contents were generally very high in the functional products as compared with levels found in conventional products (Table 4), as expected from the amounts of vitamin E or different tocopherol types commonly added to bioactive lipid-functional products.

Surprising results were found in products enriched with CLA oil, showing unusual occurrence of TGP, between 1.9 and 2.5% expressed on total lipids extracted, while oxTGM and TGD levels were low. Generally, the presence of TGP is indicative of advanced oxidation stages and is concomitant with high levels of oxTGM that form earlier in the autooxidation process through the hydroperoxide pathway (Márquez-Ruiz & Dobarganes, 2006). Thus, the typical oxidation pattern consists in the initial formation of oxTGM, mainly comprised of hydroperoxides during early oxidation, then the accumulation of sufficient amounts of oxTGM leads to formation of TGD and likewise TGP do not build up until a notable increase of TGD takes place. However, oxidation of conjugated linoleic acid seems to proceed through different mechanisms (Brimberg & Kamal-Eldin, 2003; García-Martínez, Márquez-Ruiz, Fontecha, & Gordon, 2009; Luna, De La Fuente, Salvador, & Márquez-Ruiz, 2007; Yurawecz, Delmonte, Vogel, & Kramer, 2003).

Results of oxidation parameters in the functional products at expiry times are also shown in Table 5. Among the products analysed, it is important to state that neither of the FPS samples showed oxidative changes at expiry times as compared with initial samples, in agreement with results obtained in previous works (García-Llatas et al., 2013; Soupas, Huikko, Lampi, & Piironen, 2006).

With respect to the FPω3 samples, FPω3-A, FPω3-B and FPω3-D at expiry times showed significantly increased level of oxidation as compared with initial samples, in contrast with FPω3-C, FPω3-E and FPω3-F. Specifically, a significant increase of hydroperoxides was observed in FPω-3-A, FPω-3-B and FPω-3-D, as denoted by the values obtained for PV and oxTGM, along with significant losses of

Table 3
Fatty acid composition and total lipids extracted from initial functional dairy products.[a]

FAME (wt% on oil)	FPω3-A	FPω3-B	FPω3-C	FPω3-D	FPω3-E	FPω3-F	FPCLA-A	FPCLA-B	FPCLA-C	FPCLA-D	FPSE-A	FPSE-B
C4:0	0.2 ± 0.01	0.1 ± 0.01	0.7 ± 0.04	ND	0.1 ± 0.01	0.1 ± 0.01	0.6 ± 0.04	0.3 ± 0.02	ND	ND	0.1 ± 0.01	ND
C6:0	0.1 ± 0.01	0.1 ± 0.01	0.4 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.8 ± 0.01	0.3 ± 0.01	ND	ND	0.2 ± 0.01	ND
C8:0	0.1 ± 0.00	0.1 ± 0.01	0.8 ± 0.03	0.1 ± 0.01	0.1 ± 0.00	0.1 ± 0.02	0.5 ± 0.03	ND	ND	ND	0.4 ± 0.02	ND
C10:0	0.1 ± 0.01	0.1 ± 0.01	1.6 ± 0.10	0.9 ± 0.08	0.1 ± 0.01	0.1 ± 0.01	1.0 ± 0.10	0.6 ± 0.03	ND	ND	0.3 ± 0.01	ND
C10:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C12:0	0.2 ± 0.01	0.3 ± 0.01	2.3 ± 0.20	1.0 ± 0.07	0.3 ± 0.02	0.4 ± 0.03	1.2 ± 0.07	0.7 ± 0.03	0.1 ± 0.00	0.1 ± 0.01	0.5 ± 0.01	ND
C13:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C14:0	0.5 ± 0.03	1.2 ± 0.10	9.5 ± 0.88	5.3 ± 0.42	1.2 ± 0.08	0.5 ± 0.02	3.7 ± 0.21	1.9 ± 0.10	ND	ND	1.4 ± 0.05	ND
C15:0	ND	ND	0.1 ± 0.01	ND	ND	ND	0.1 ± 0.01	0.1 ± 0.00	ND	ND	ND	ND
C16:0	9.4 ± 1.10	10.1 ± 0.71	31.9 ± 1.02	26.7 ± 0.91	9.0 ± 0.32	6.7 ± 0.20	11.7 ± 0.90	7.8 ± 0.41	1.6 ± 0.11	6.0 ± 0.22	10.9 ± 0.75	6.3 ± 0.39
C16:1	0.1 ± 0.01	ND	2.3 ± 0.11	2.0 ± 0.10	0.1 ± 0.00	0.1 ± 0.03	0.5 ± 0.03	0.5 ± 0.00	ND	ND	1.1 ± 0.04	1.0 ± 0.05
C18:0	8.7 ± 0.72	6.0 ± 0.33	12.1 ± 0.59	9.0 ± 0.35	7.4 ± 0.51	3.3 ± 0.28	5.5 ± 0.27	3.5 ± 0.31	2.5 ± 0.22	3.8 ± 0.18	5.1 ± 0.21	5.4 ± 0.14
C17:0	0.1 ± 0.03	0.1 ± 0.02	0.2 ± 0.00	ND	ND	ND	0.2 ± 0.01	ND	ND	ND	0.1 ± 0.00	ND
C17:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:1	65.0 ± 1.50	63.7 ± 1.65	28.3 ± 0.73	44.6 ± 1.05	68.8 ± 2.10	24.2 ± 1.10	17.5 ± 1.10	15.6 ± 0.75	14.7 ± 0.87	16.2 ± 1.02	57.9 ± 1.34	68.1 ± 1.47
C18:2 9*c*,12*c*	12.1 ± 0.55	10.4 ± 0.41	4.9 ± 0.30	3.7 ± 0.22	7.9 ± 0.44	62.7 ± 2.07	1.3 ± 0.08	1.4 ± 0.09	0.4 ± 0.02	0.8 ± 0.02	16.9 ± 0.76	14.2 ± 0.91
C18:2 9*c*,11*t*	ND	ND	ND	ND	ND	ND	26.6 ± 1.00	29.2 ± 1.08	38.0 ± 0.99	34.1 ± 0.77	ND	ND
C18:2 10*t*,12*c*	ND	ND	ND	ND	ND	ND	25.6 ± 0.86	30.1 ± 0.96	37.8 ± 0.84	33.1 ± 0.93	ND	ND
C18:3 *n*-3	0.4 ± 0.02	0.1 ± 0.01	0.2 ± 0.01	0.1 ± 0.03	0.1 ± 0.02	0.2 ± 0.02	0.1 ± 0.01	0.1 ± 0.02	ND	ND	0.4 ± 0.02	2.3 ± 0.10
C20:0	ND	ND	0.1 ± 0.00	0.1 ± 0.01	ND	ND	0.1 ± 0.00	0.1 ± 0.01	ND	ND	ND	ND
C20:5 *n*-3	1.4 ± 0.01	1.5 ± 0.12	1.4 ± 0.15	1.6 ± 0.15	1.4 ± 0.03	0.4 ± 0.00	ND	ND	ND	ND	ND	ND
C22:6 *n*-3	2.3 ± 0.01	2.7 ± 0.08	2.0 ± 0.11	2.0 ± 0.27	0.6 ± 0.01	0.2 ± 0.01	ND	ND	ND	ND	ND	ND
∑SFA	17.4	18.1	59.7	43.2	18.3	11.3	25.4	15.2	4.2	9.9	19.0	11.7
∑MUFA	65.1	66.7	30.6	46.6	68.9	24.3	18.0	16.1	14.7	16.2	59.0	69.1
∑PUFA	16.2	14.7	7.5	7.5	10.0	63.5	53.6	60.8	76.2	68.0	17.3	16.5
∑*n*-3	4.1	4.3	3.6	3.7	2.1	0.8	0.1	0.1	ND	ND	ND	ND
∑CLA	ND	ND	ND	ND	ND	ND	52.2	59.3	75.8	67.2	ND	ND
Extracted lipids (wt% on product)	3.0 ± 0.15	2.6 ± 0.23	2.8 ± 0.11	3.1 ± 0.34	1.9 ± 0.18	1.7 ± 0.22	1.0 ± 0.20	1.6 ± 0.15	2.0 ± 0.25	1.5 ± 0.10	5.1 ± 0.37	4.0 ± 0.30

[a] Abbreviations are: FAME, fatty acid methyl esters; CLA, conjugated linoleic acids; FPω3, functional dairy product with omega-3 fatty acids; FPCLA, functional dairy product with conjugated linoleic acid; FPSE, functional dairy product with stanol esters; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CLA, conjugated linoleic acid; ND, not detected. Data are means ± SD ($n = 3$).

Table 4
Oxidation parameters in lipids extracted from conventional dairy products at initial time.[a]

Parameter	PM-A	PM-B	UHTM-A	UHTM-B	FM-A	FM-B	EM-A	EM-B	POM-A		POM-B	
									Free	Encapsulated	Free	Encapsulated
PV	0.8 ± 0.34	0.7 ± 0.40	1.0 ± 0.36	0.9 ± 0.28	0.6 ± 0.44	0.7 ± 0.38	0.9 ± 0.26	1.0 ± 0.35	6.9 ± 0.57^{a}	1.3 ± 0.20^{b}	7.5 ± 0.61^{a}	1.5 ± 0.29^{b}
TG (mg g^{-1})												
- OxTGM	ND	ND	ND	ND	ND	ND	1.8 ± 0.30	2.1 ± 0.28	16.1 ± 1.36^{a}	1.5 ± 0.20^{b}	10.4 ± 2.17^{a}	2.3 ± 0.25^{b}
- TGD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
- TGP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
TOC (mg kg^{-1})												
- α	28.6 ± 4.16	29.2 ± 4.18	28.4 ± 3.06	27.0 ± 4.12	26.1 ± 4.10	21.9 ± 5.14	37.9 ± 5.21	32.6 ± 4.16	20.5 ± 5.09	31.7 ± 6.86	26.1 ± 4.32	27.3 ± 2.99
- β	27.2 ± 4.62	ND	26.4 ± 4.28	ND	9.8 ± 3.33	27.0 ± 5.69	ND	ND	ND	ND	ND	ND
- γ	ND	21.3 ± 3.14	ND	17.6 ± 2.05	ND	ND	ND	ND	32.1 ± 3.22^{a}	44.8 ± 4.70^{b}	18.4 ± 2.56^{a}	26.0 ± 3.01^{b}
- δ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	55.9 ± 6.22	50.4 ± 5.22	54.7 ± 5.26	44.6 ± 4.60	35.9 ± 5.28	48.9 ± 7.67	37.9 ± 5.21	32.6 ± 4.16	52.6 ± 4.31^{a}	76.5 ± 5.03^{b}	44.5 ± 4.67^{a}	53.3 ± 3.28^{b}

[a] Abbreviations: PM, pasteurised whole milk; UHTM, ultra-high temperature-treated whole milk; FM, fermented milk; EM, evaporated whole milk; POM, powdered whole milk; PV, peroxide value; TG, triacylglycerols; OxTGM, oxidised triacylglycerol monomers; TGD, triacylglycerol dimers; TGP, triacylglycerol polymers; TOC, tocopherols; ND, not detected. Data are means ± SD ($n = 3$); different superscript letters in POM samples indicate significant differences between free and encapsulated lipid fractions ($p < 0.05$).

tocopherols (21–35%), especially α-tocopherol. Nor the fatty acid composition, nor the tocopherol contents, nor the initial oxidation levels accounted for the differences found among FPω3 samples. In samples FPω3-C and FPω3-E, fish oil was employed as micro-encapsulated fish oil (information provided by the companies) rather than emulsified directly in the skimmed milk, which could have contributed to increasing protection towards oxidation. In fact, microencapsulation enables supplementation of functional foods with higher amounts of fish oil by virtue of solubility enhancement, masking of unpleasant fishy taste and odour and protection from pro-oxidant agents (Beindorff, Zuidam, Zuidam, & Nedovic, 2010).

Among the products enriched with CLA, FPCLA-A and FPCLA-D showed significantly higher total oxidation compounds at expiry times than the initial samples. In contrast with results obtained for FPω-3 samples, values of PV and oxTGM remained low in FDPCLA-A and FPDCLA-D. Therefore, in such samples the increase of total oxidation compounds during storage was exclusively attributable to significant formation of TGP. Tocopherol decreases were also significant (about 14%) but lower than in the case of FPω-3 samples and only due to δ-tocopherol losses.

Fig. 1 shows exclusion chromatograms of the polar fractions of samples FPω3-A and FPCLA-A at the expiry dates, illustrating their different distribution of oxidation compounds. For similar total oxidation levels (Fig. 1), oxTGM were major compounds in FPω3-A, while TGP were predominant in FPCLA-A. The early retention time at which TGP started to elute, implying unusually high molecular weight or size, indicates that formation of very complex molecules was favoured even under the mild shelf-life conditions recommended by producers, room temperature in the case of FPCLA-A. This large degree of polymerisation has already been observed in a previous work in which conjugated methyl linoleate was used as a model compound (Luna et al., 2007).

It is difficult to ascertain the main sources of lipid oxidation in commercial functional dairy products containing ω-3 PUFAs or CLA, which include a complex mixture of major ingredients, oils, mineral salts and vitamins. Most of these products are oil-in-water emulsions highly susceptible to oxidation where the oil-water interface greatly affects the distribution of the compounds that impact oxidation. For example, the right selection of antioxidants is of the utmost importance to ensure that they are located at the major site of the lipid oxidation reactions. It is also clear that the initial quality of the oils used as well as the protection against pro-oxidants agents in the emulsion systems are determinant parameters for the oxidative stability of dairy products (Jacobsen, 2010). A large number of studies have been carried out by Let et al. (2003, 2004, 2005, 2007) to evaluate the effects of ω-3 PUFAs addition to commercial milk as substrate; the authors have consistently found significant but low increases of PV in milk emulsions containing fish oil.

Regarding dairy products containing CLA, most published studies have shown that processing parameters such as heating or fermentation had no significant effect on the CLA levels (Bisig, Eberhard, Collomb, & Rehberger, 2007). However, loss of CLA based on gas-chromatography analysis of FAME derivatives is not sensitive enough since a significant decrease of polyunsaturated fatty acids is not detected until well within the advanced oxidation stage (Angulo, Romera, Ramírez, & Gil, 1998; García-Martínez, Holgado, Velasco, & Márquez-Ruiz, 2012). Even so, significant losses of CLA isomers have been found after HTST pasteurisation (up to 10%) and further losses upon refrigerated storage (up to 6%) (Campbell, Drake, & Larick, 2003). Moreover, losses of CLA as high as 22% have recently been reported in an enriched milk after UHT treatment, while only slight increases of hydroperoxides were detected (Martínez-Monteagudo et al., 2015). Since hydroperoxides

Table 5
Oxidation parameters in lipids extracted from functional dairy products at initial and expiry times.[a]

Parameter	FPω3-A	FPω3-B	FPω3-C	FPω3-D	FPω3-E	FPω3-F	FPCLA-A	FPCLA-B	FPCLA-C	FPCLA-D	FPSE-A	FPSE-B
Initial time												
PV (meq O_2 kg^{-1})	8.2 ± 2.26[a]	6.7 ± 2.22[a]	3.9 ± 2.34	8.8 ± 2.41[a]	9.1 ± 2.61	9.6 ± 2.48	6.6 ± 3.50	7.3 ± 2.62	6.0 ± 2.81	7.9 ± 2.61	4.9 ± 3.49	3.3 ± 2.18
TG (mg g^{-1})												
- OxTGM	9.1 ± 1.12[a]	6.1 ± 1.14[a]	5.2 ± 1.12	9.2 ± 1.15[a]	9.3 ± 1.19	9.0 ± 1.22	8.5 ± 1.13	8.0 ± 1.28	7.0 ± 1.03	5.2 ± 1.23	6.9 ± 1.30	5.0 ± 0.41
- TGD	8.5 ± 1.11	3.2 ± 0.30	1.1 ± 0.10	7.1 ± 1.10	6.9 ± 1.08	6.1 ± 1.17	3.2 ± 0.41	2.8 ± 0.31	3.5 ± 0.20	2.5 ± 0.88	3.4 ± 0.37	ND
- TGP	1.9 ± 0.18	ND	ND	ND	1.7 ± 0.20	ND	24.3 ± 1.35[a]	21.2 ± 2.07	19.3 ± 2.12	24.9 ± 1.91[a]	ND	ND
TOC (mg kg^{-1})												
- α	532.0 ± 15.52[a]	445.6 ± 14.54[a]	406.1 ± 21.37	335.1 ± 12.86[a]	524.4 ± 14.62	526.4 ± 15.04	65.1 ± 4.13	93.4 ± 5.44	78.9 ± 5.99	28.1 ± 2.14	12.3 ± 1.55	220.1 ± 10.34
- β	34.5 ± 2.20	29.0 ± 3.03	25.1 ± 3.08	7.7 ± 1.27	10.3 ± 1.04	36.1 ± 4.39	13.1 ± 1.22	22.1 ± 1.30	ND	ND	ND	ND
- γ	147.5 ± 8.81	102.8 ± 5.19	92.0 ± 6.04	26.7 ± 0.91	166.1 ± 6.26	44.7 ± 4.46	312.0 ± 13.74	593.1 ± 15.61	511.6 ± 20.11	324.0 ± 17.22	116.2 ± 7.56	55.3 ± 3.39
- δ	43.8 ± 2.45	40.1 ± 2.66	337.0 ± 18.11	45.7 ± 1.62	84.0 ± 5.49	3.2 ± 0.40	250.3 ± 8.15[a]	318.6 ± 11.01	268.0 ± 7.44	215.2 ± 12.99[a]	35.0 ± 1.18	19.9 ± 2.05
Total	757.6 ± 18.15[a]	617.3 ± 15.96[a]	860.2 ± 28.82	396.9 ± 13.06[a]	784.7 ± 16.86	610.4 ± 16.29	640.4 ± 16.55[a]	1027.4 ± 19.90	855.0 ± 22.26	567.3 ± 21.68[a]	163.5 ± 7.81	295.3 ± 11.07
Expiry time												
PV (meq O_2 kg^{-1})	65.9 ± 4.16[b]	26.0 ± 3.01[b]	4.1 ± 3.22	44.5 ± 5.01[b]	10.9 ± 3.87	11.5 ± 4.01	7.2 ± 5.18	8.5 ± 2.09	7.1 ± 2.30	8.3 ± 3.99	3.8 ± 2.98	4.2 ± 2.87
TG (mg g^{-1})												
- OxTGM	41.1 ± 2.88[b]	19.1 ± 1.55[b]	6.1 ± 1.30	34.5 ± 1.64[b]	10.2 ± 2.30	11.2 ± 2.37	8.0 ± 1.17	9.3 ± 1.39	8.3 ± 1.88	10.4 ± 1.88	5.7 ± 1.51	7.3 ± 2.06
- TGD	9.9 ± 1.05	3.2 ± 0.29	1.0 ± 0.36	7.9 ± 0.87	7.9 ± 1.97	7.4 ± 2.26	3.5 ± 0.50	3.7 ± 1.23	3.6 ± 0.65	5.0 ± 1.01	ND	ND
- TGP	2.1 ± 0.14	ND	ND	ND	1.6 ± 0.47	ND	48.9 ± 3.22[b]	25.9 ± 4.47	21.9 ± 2.94	32.8 ± 2.98[b]	ND	ND
TOC (mg kg^{-1})												
- α	332.6 ± 11.41[b]	346.2 ± 13.10[b]	399.0 ± 18.21	198.7 ± 9.31[b]	511.7 ± 21.85	490.4 ± 23.26	60.0 ± 2.91	82.5 ± 7.56	75.6 ± 7.01	21.8 ± 2.31	15.9 ± 1.22	199.6 ± 12.58
- β	29.1 ± 2.87	25.1 ± 1.04	20.8 ± 5.01	ND	8.7 ± 2.09	25.0 ± 5.22	10.7 ± 0.53	23.4 ± 2.56	ND	ND	ND	ND
- γ	93.0 ± 7.18	80.9 ± 4.59	91.3 ± 5.93	20.0 ± 1.22	146.3 ± 9.11	39.9 ± 4.87	283.5 ± 10.18	512.7 ± 20.31	498.8 ± 24.87	309.9 ± 18.02	136.1 ± 8.36	47.5 ± 3.70
- δ	40.2 ± 3.04	36.1 ± 2.89	330.2 ± 24.60	38.9 ± 3.03	73.8 ± 8.62	2.4 ± 0.56	201.4 ± 9.31[b]	294.4 ± 19.89	211.9 ± 10.20	154.2 ± 12.11[b]	29.7 ± 1.45	21.2 ± 2.44
Total	494.5 ± 14.11[b]	487.0 ± 14.22[b]	853.1 ± 33.76	259.6 ± 9.87[b]	729.1 ± 18.03	584.8 ± 23.60	551.7 ± 14.11[b]	990.82 ± 33.22	827.0 ± 19.98	486.1 ± 19.47[b]	170.3 ± 6.62	286.9 ± 12.20

[a] Abbreviations are: FPω3, functional dairy product with omega-3 fatty acids; FPCLA, functional dairy product with conjugated linoleic acid; FPSE, functional dairy product with stanol esters; PV, peroxide value; TG, triacylglycerols; OxTGM, oxidised triacylglycerol monomers; TGD, triacylglycerol dimers; TGP, triacylglycerol polymers; TOC, tocopherols; ND, not detected. Data are means ± SD ($n = 3$); different superscript letters indicate significant differences between samples at initial and expiry times ($p < 0.05$).

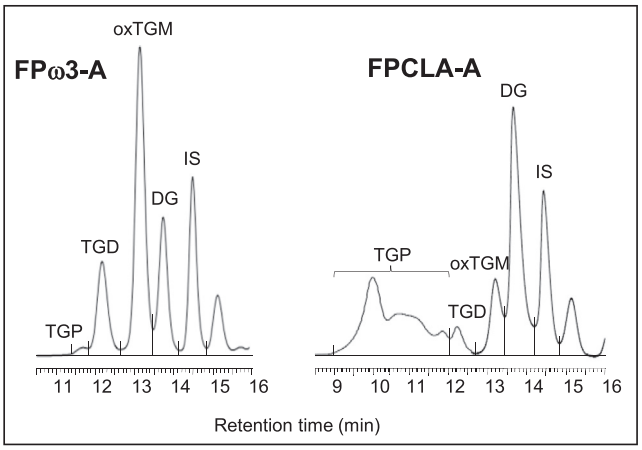

Fig. 1. High-performance size-exclusion chromatograms of polar fractions of lipids extracted from a functional dairy product with omega-3 fatty acids (FPω3-A; left) and a functional dairy product with conjugated linoleic acid (FPCLA-A; right) at the expiry dates. Abbreviations: TGP, triacylglycerol polymers; TGD, triacylglycerol dimers; oxTGM, oxidised triacylglycerol monomers; DG, diacylglycerols and IS, internal standard (monostearin).

are only minor products in the oxidation of CLA (Luna et al., 2007), it is clear that the determination of PV provided misleading results.

4. Conclusions

The method proposed to evaluate major, non-volatile oxidised triacylglycerols in dairy products has provided complete and quantitative information on the oxidation state, including individual quantitation of primary and final oxidation products. Application of this method to commercial dairy products at initial and expiry dates under shelf-life conditions has provided complete data on oxidative changes.

In contrast to the high stability against oxidation of conventional dairy products, half of the analysed products enriched with ω-3 fatty acids or CLA showed significantly increased total oxidation compounds. For the former, changes were attributable to hydroperoxide formation, clearly denoted by increases of peroxide values and oxidised triacylglycerol monomers. However, in the case of CLA products, formation of polymers is prevalent and would have gone undetected if a global measurement of the oxidation state had not been carried out. This is of utmost importance since PV, which only enables detection of hydroperoxide formation, is at present the only determination included in quality specifications of CLA-rich ingredients used in commercialised dairy products. Polymer determination has therefore proven to be a highly valuable technique and could be an alternative routine method to evaluate oxidation of CLA-rich products.

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3.3.- Efecto del tratamiento de esterilización clásica en fórmulas líquidas infantiles modelo con base láctea, de inicio y de continuación, sobre la oxidación lipídica.

Effect of classic sterilization on lipid oxidation in model liquid milk-based infant and follow-on formulas.

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Resumen:

El objetivo de este estudio fue evaluar el efecto de la esterilización clásica sobre la oxidación lipídica en la leche de fórmula de inicio y en las fórmulas de continuación, analizando la formación de triglicéridos monómeros oxidados y dimeros. Para obtener un perfil de ácidos grasos de acuerdo con las normativas de la UE, se utilizaron sistemas modelo con componentes y en proporciones similares a aquellas que normalmente se encuentran en fórmulas comercializadas. Se utilizó una mezcla de aceite de girasol alto-oleico, aceite de colza bajo-erúcico y aceite de pescado para conseguir un perfil de ácidos grasos que se ajustase a las especificaciones de la regulación de la UE. Con fines comparativos, algunas muestras fueron preparadas sólo con aceite de girasol alto-oleico o bien sólo con aceite de pescado y otras sin la fracción proteica y adicionando Tween-20. La cuantificación total de los triglicéridos oxidados proporcionó una información completa sobre el estado oxidativo y mostró claras ventajas frente a otros métodos utilizados, por ejemplo la cuantificación de la pérdida de ácidos grasos poliinsaturados o el índice de peróxidos. Los resultados mostraron que el tratamiento térmico utilizado en la esterilización no condujo a una oxidación lipídica significativa, sin embargo, la concentración de tocoferoles disminuyó significativamente. La marcada pérdida de tocoferoles encontrada en las fórmulas sin fracción proteica, junto con las concentraciones significativamente más bajas de tocoferoles en las fórmulas infantiles de inicio (80% proteínas de suero lácteo) en comparación con los preparados de continuación (80% caseinato), refleja el efecto protector de la fracción protéica, especialmente del caseinato de sodio.

Research Article

Effect of classic sterilization on lipid oxidation in model liquid milk-based infant and follow-on formulas

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The objective of this study was to evaluate the effect of classic sterilization on lipid oxidation of liquid infant and follow-on formulas by analyzing formation of oxidized and dimeric TAGs. Model systems containing similar components and proportions to those normally found in manufactured samples and a mixture of high-oleic sunflower oil, rapeseed oil, and fish oil were used to obtain a fatty acid composition profile in accordance with the EU regulations. For comparative purposes, some samples were prepared with high-oleic sunflower or fish oil and others without the protein components and added Tween-20. Quantification of total oxidized TAGs provided complete information of the oxidation state and showed clear advantages versus the other methods used, i.e., loss of PUFA and peroxide value. The results showed that the heat treatment used for sterilization did not lead to significant lipid oxidation, but the tocopherol concentration decreased significantly. The marked tocoherol losses found in protein-free formulas together with the significantly lower tocopherol concentrations in infant formulas (80% whey in protein fraction) compared to follow-on formulas (80% caseinate in protein ratio) showed the protective effect of the protein fraction, specially sodium caseinate.

Practical applications: This study provides useful information on the utility of different methods used to evaluate oxidation in infant and follow-on formulas. Quantification of total oxidized TAGs standed out because it is a direct and sensitive method and provides complete information at any stage of the oxidative process. Also, this study shows that important decreases of tocopherols may occur during formula processing and special cautions should therefore be taken during storage and commercialization to avoid additional antioxidant losses.

Keywords: Infant formula / Lipid oxidation / Oxidized TAGs / Sterilization / Tocopherols

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1 Introduction

Lipid oxidation is one of the most relevant alterations that occur during food processing and storage due to the development of odors and flavors that are responsible for rancidity and greatly influence consumer acceptance [1]. Formation of non-volatile oxidation compounds can have negative effects on nutritional food quality and food safety. In this context, an aspect of the utmost concern nowadays is the negative physiological implications of oxidized lipids, particularly on cellular aging mechanisms and on the development of degenerative diseases like cardiovascular disease and cancer [2–5].

Autoxidation is the main oxidation mechanism of food lipids. It is an autocatalytic process of chain reactions occurring throughout formation of free radicals [1]. The diverse final distribution of oxidation products depends on many variables that determine the relative speed of reactions between radicals and the subsequent oxidation of the low stability compounds that are formed. Among the variables that may exert an important influence upon the development of oxidation, temperature, oxygen availability, the presence of antioxidants, and pro-oxidants and the degree of unsaturation of fatty acids are prominent.

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Abbreviations: HPSEC, high-performance size-exclusion chromatography; **OSI**, Oil Stability Index; **SMUF**, simulated milk ultrafiltrate; **SPE**, solid-phase extraction

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The complexity of oxidation greatly increases in dispersed systems such as emulsions, mainly due to the discontinuity of the lipid phase [6] and the effect of additional variables, namely, water activity, homogenization conditions, pH and fat globule size, among others [7–9].

Infant formula emulsions are among the most interesting regarding lipid oxidation. Firstly, they can be the only source of nutrition for newborn infants. Secondly, the joint addition of prooxidants (minerals such as iron) and oils with a high content of nutritionally important but oxidizable PUFA such as long-chain omega-3 PUFA, makes infant formulas particularly susceptible to oxidative modifications [10–12]. However, lipid oxidation in infant formulas has been poorly studied. In this context, published research has focused on oxidative modifications of infant formulas occurring during storage, usually evaluated by classic indirect methods [13–15] or by the quantitation of residual tocopherols [13, 16–18]. Results are often confusing and contradictory, mainly due to the methods used, which only provide information on partial aspects of the oxidation process. Classic sterilization is one of the thermal processes most commonly used in the manufacture of infant formulas. It can be achieved by applying the proper combinations of heat and time to guarantee microbiological safety but the high temperatures involved could induce oxidation reactions. However, regarding the influence of sterilization treatments on lipid oxidation of infant formula, no published work has been found.

The aim of this study was to determine the effect of classic in-bottle sterilization treatment on lipid oxidation in model systems simulating liquid milk-based infant and follow-on formulas. For this purpose, a novel method consisting of the analysis of the total fraction of non-volatile lipid oxidation products and their distribution in oligomers, dimers, and monomers was applied for evaluation of lipid oxidation. Besides, other conventional methods were applied, namely, the loss of unsaturated fatty acids, the peroxide value, and the loss of tocopherols.

2 Materials and methods

2.1 Samples

Model systems simulating milk-based infant and follow-on formulas were prepared with components and proportions similar to those normally found in manufactured products.

The formulation (w/v) was as follows: 2.5% milk proteins, 8.0% lactose and 3.0% oil in a simulated milk ultrafiltrate (SMUF) solution. Milk proteins used were sodium caseinate from bovine milk (Sigma-Aldrich, St Luis, MO, USA) and whey protein isolate BiPRO (Davisco, Le Sueur, MN, USA). D-lactose monohydrate was used (Sigma-Aldrich, St Luis, MO, USA). SMUF was prepared as follows [19]: 0.158% KH_2PO_4, 0.120% $K_3Citr{\cdot}H_2O$, 0.179% $Na_3Citr{\cdot}2H_2O$, 0.018% K_2SO_4, 0.132% $CaCl_2{\cdot}2H_2O$, 0.065% $MgCl_2{\cdot}6H_2O$, 0.030% K_2CO_3, y 0.060% KCl in distilled water (all % w/w). As for milk proteins, the sodium caseinate-to-whey protein ratio was 1:4 for infant formulas and 4:1 for follow-on formulas. A mixture of high-oleic sunflower oil (Koipesol, Sevilla, Spain), rapeseed oil (low-erucic rapeseed oil, Interfat S.A., Barcelona, Spain) and fish oil (ROCHE, Barcelona, Spain) (65/30/5 v/v/v) here called ''oil mixture'' was used to obtain a fatty acid composition profile in accordance with the Spanish Regulations [20]. For comparative purposes, some samples were prepared exclusively with high-oleic sunflower or fish oil, and others without the protein components and added 0.5% Tween-20 (''protein-free formulas'') [21]. Also, $FeCl_3$ (100 μM) and ascorbate (200 μM) were added to selected samples as a prooxidant agent [22].

The processing of the formulas was similar to that used in the food industry [23]. Briefly, a coarse emulsion was prepared in a mixer for 1 min (Ultra Turrax), followed by homogenization at 50 MPa (2 passes) in a high-pressure laboratory homogenizer Rannie 8.30 (APV, Madrid, Spain). Then, 90 mL-samples were placed into 100 mL-glass bottles, sealed and heated at 120°C for 15 min. After cooling, EDTA (100 μM) was added and the samples were frozen at −40°C until analyses. All chemicals and reagents were obtained from Sigma (Sigma–Aldrich, St Luis, MO, USA). All samples were prepared in triplicate.

2.2 Lipid extraction

Lipid fraction was extracted from infant formulas in separatory funnels by three consecutive liquid-liquid extractions with a mixture of ethanol, diethyl ether and pentane (1:5:5 v/v/v). The upper layer was filtered through sodium sulfate and evaporated to dryness in a rotary evaporator. Oil dried to constant weight were stored in amber glass vials, exposed to a stream of nitrogen and frozen at −20°C until analysis.

2.3 Fatty acid composition

Fatty acid profile of model infant formulas was determined by GC of FAME. The FAME were prepared by base-catalyzed methanolysis of the extracted lipids using 2 N KOH in methanol as described by IUPAC [24, 25]. The FAME were analyzed on an HP-6890 (Hewlett Packard, Avondale, PA, USA) with a FID detector and an HP Innowax capillary column (30 m × 0.25 mm id, 0.25 μm film thickness). The temperature program used was: initial temperature of 180°C held for 2 min, then raised to 230°C at a rate of 3°C/min and held for 20 min. Hydrogen was the carrier gas at a flow rate of 1 mL/min with a split ratio of 1:40. The injector and detector temperature was 250°C.

2.4 Peroxide value

Peroxide value was determined by the standard iodometric method [26]. Samples of 500 mg lipids and a 1×10^{-2} mol/L $Na_2S_2O_3$ solution were used.

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2.5 Quantitation of oxidation compounds

Quantitative analysis of total non-volatile oxidation compounds was carried out by separation of polar compounds by solid-phase extraction (SPE) and subsequent analysis by high-performance size-exclusion chromatography (HPSEC) with refractive index detection according to the method of Márquez-Ruiz et al. [27].

2.5.1 Separation of polar compounds by SPE

A volume of 2 mL of a hexane solution containing 50 mg of extracted oil and 1 mg of monostearin (Sigma, St. Louis, Mo., USA), used as an internal standard (IS), was separated by SPE into two fractions in a Waters Sep-pak silica cartridge (1 g of silica, Waters, Milford, MA, USA). The first fraction comprises the unoxidized TAGs and was eluted with 15 mL of hexane:diethyl ether (90:10, v/v). The second fraction was eluted with 25 mL of diethyl ether and contains the total non-volatile oxidation compounds, lipid hydrolysis products, i.e., DAGs and free fatty acids, and the polar unsaponifiable matter. Thus, the lipid oxidation products are separated as compounds with higher polarity than that of the unoxidized TAG molecules. After removal of the solvent in a rotary evaporator, the polar fraction was dissolved with 1 mL of diethyl ether. Efficiency of the separation was checked by TLC using hexane/diethyl ether/acetic acid (80:20:1, v/v/v) for development of plates and exposure to iodine vapor to reveal the spots.

2.5.2 Analysis by HPSEC

The fraction of polar compounds was analyzed in an HPSEC chromatograph equipped with a Rheodyne 7725i injector with 10 μL sample loop, a Knauer 1200 HPLC pump (Knauer, Germany) and an HP 1037 A refractive index detector (Agilent Technologies, Palo Alto, CA). The separation was performed on two 100 and 500 Å Ultrastyragel columns (25 cm × 0.77 cm I.D.) packed with porous, highly cross-linked styrene-divinylbenzene copolymers (film thickness 10 μm) (Agilent Technologies) connected in series, with tetrahydrofuran (1 mL/min) as the mobile phase. The peaks resolved by HPSEC correspond to TAG polymers, TAG dimers, oxidized TAG monomers, DAGs, monostearin, and a final peak corresponding to the free fatty acids and the polar unsaponifiable matter. This methodology was described in detail, including precision, accuracy, and recovery data, in a previous publication [27].

2.6 Determination of tocopherols

Analysis of tocopherols was performed by normal-phase HPLC with fluorescence detection according to IUPAC Standard Method 2.411 [28].

2.7 Oxidative Stability Index (OSI)

OSI of oils used for preparation of model infant formulas was determined with a 679 Rancimat device (Metrohm, Glostrup, Denmark) [29]. The tests were carried out with 2 g of oil at 100°C and 20 L/h of air.

2.8 Statistical analysis

Analytical determinations of initial oils were carried out in triplicate and results were expressed as mean values followed by SDs. Model infant and follow-on formulas were analyzed in triplicate samples and results were expressed as mean values followed by SDs. Comparisons between means were made by applying the non-parametric Kruskal–Wallis test, when comparing K independent samples, and the Mann–Whitney *U* test, when comparing two independent samples, using SPSS Statistics version 17.0 (SPSS Inc., Ireland). Significant differences were established at $p < 0.05$.

3 Results and discussion

3.1 Characterization of initial oils

The fatty acid profiles of the oils used and the oil mixture prepared to simulate the lipid composition of manufactured infant formulas are shown in Table 1. The oil mixture of high oleic sunflower oil (65%), low erucic rapeseed oil (30%) and fish oil (5%) meets all the requirements specified in the Spanish Royal Decree 867/2008, through which the Technical-Sanitary Regulations specific to infant and follow-on formulas were approved [20]. This Royal Decree incorporates the European Union Directives 2006/141/CE and 1999/21/CE and establish limitations for lauric, myristic, erucic and 20 carbon-chain fatty acids, as well as linoleic and α-linolenic acids content ranges and ratio. Such regulations do not establish any differentiation in fatty acid composition requirements between infant and follow-on formulas [20].

Table 2 shows the results for peroxide values, OSI and major natural antioxidants (tocopherols) in the oils used for preparation of infant and follow-on formulas. Peroxide value data were those expected for refined oils (below 10 meq/kg oil). The fish oil contained the highest level of peroxides, being the most susceptible to oxidation among the oils used due to its high PUFA content (Table 1), specifically DHA (DHA or C22:6 = 13.0 ± 0.23% of methyl esters). The fish oil showed an OSI value of 3.2 hours, while the high oleic sunflower oil was the most stable, with an OSI of 23.0 hours and both the low erucic rapeseed oil and the mixture oil prepared showed intermediate values. Table 2 also shows quantitative results for the oxidation level of initial oils, following the method combining SPE and HPSEC to determine the total non-volatile oxidation compounds and their distribution in oxidized TAG monomers, dimers and oligomers [27]. The results obtained showed oxidation levels commonly

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Table 1. Major fatty acid composition of the oils used in the preparation of model formulas (% of methyl esters)

Fatty acid	High-oleic sunflower oil	Low erucic acid rapeseed oil	Fish oil	Oil mixture
C14:0			5.8 ± 0.17	0.3 ± 0.03
C16:0	4.3 ± 0.06	5.2 ± 0.07	16.3 ± 0.21	4.1 ± 0.05
C18:0	3.9 ± 0.07	1.7 ± 0.08	3.5 ± 0.03	2.9 ± 0.03
C20:0		0.5 ± 0.03	0.2 ± 0.01	0.2 ± 0.01
C22:0				
C14:1				
C16:1		0.4 ± 0.02	5.8 ± 0.04	0.3 ± 0.01
C18:1	72.4 ± 0.65	58.3 ± 0.77	20.1 ± 0.34	65.6 ± 0.81
C20:1		1.4 ± 0.01	1.8 ± 0.02	0.4 ± 0.02
C22:1		1.2 ± 0.02	0.2 ± 0.01	0.3 ± 0.01
C18:2	18.2 ± 0.33	22.3 ± 0.24	13.5 ± 0.10	19.4 ± 0.15
C18:3		8.8 ± 0.06	2.0 ± 0.05	2.7 ± 0.05
C18:4				
C20:5			8.7 ± 0.07	0.4 ± 0.01
C22:6			13.0 ± 0.23	0.6 ± 0.02
Total				
SFA	8.2 ± 0.09	7.4 ± 0.11	28.9 ± 0.27	7.5 ± 0.07
MUFA	72.4 ± 0.65	61.3 ± 0.77	27.9 ± 0.34	66.6 ± 0.81
PUFA	18.2 ± 0.32	31.1 ± 0.25	37.2 ± 0.26	23.1 ± 0.15

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
Data are expressed as Mean ± SD, $n = 3$.

found in refined oils. The total tocopherol contents were similar for the three oils, whereas their distribution in different tocopherol types was quite different. The fish oil had a slightly higher total tocopherol concentration, being remarkable its content of gamma- and alpha-tocopherol. In the case of high oleic sunflower oil, the tocopherols essentially consisted of alpha, while the low erucic rapeseed oil had the highest and lowest concentrations of gamma- and alpha-tocopherol, respectively.

3.2 Evaluation of oxidation in oils extracted from model infant and follow-on formulas

One of the methods used to evaluate the level of lipid oxidation in oils and in foods in general is the analysis of changes in the fatty acid profile by GC despite its low sensitivity [1]. The fatty acids most susceptible to oxidation are PUFA, and their decrease in infant and follow-on formulas implies a loss of nutritional value with negative consequences in infants, whose diet requires certain minimum level of such essential nutrients. After examining changes in PUFA concentration in all samples by GC, only significant differences were obtained in the protein-free formulas after heat treatment. Therefore, Fig. 1 only includes changes found in oils extracted from the samples prepared without the protein fraction.

Results presented in Fig. 1 indicated, firstly, that milk proteins exerted an important protective action on lipids in terms of oxidation. Multiple pathways may be involved in such effects, including inactivation of ROS, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties of food systems [30]. Furthermore, it has been recently reported that unadsorbed proteins exert greater antioxidative effects than do adsorbed proteins [31]. In the particular case of casein, the ability of phosphorylated groups to chelate pro-oxidant metals ions may explain this inhibition, but free-radical-scavenging activity could also be involved [32].

Secondly, the differences between samples with and without Fe were lower than expected, and only significant in the high-oleic sunflower oil samples. The prooxidant effect of the iron/ascorbate complex is based on the capacity of

Table 2. Oxidation parameters and tocopherol contents of the oils used in the preparation of model formulas

	High-oleic sunflower oil	Rapeseed oil	Fish oil	Oil mixture
Peroxide value (meq O_2/kg)	5.2 ± 0.1	7.8 ± 0.5	9.2 ± 0.2	6.1 ± 0.1
Oxidation compounds (%):				
Total	1.5 ± 0.1	2.5 ± 0.1	2.0 ± 0.1	1.9 ± 0.1
Oxidized TAG monomers	1.21 ± 0.08	2.07 ± 0.09	1.74 ± 0.08	1.48 ± 0.06
TAG dimers	0.32 ± 0.03	0.40 ± 0.01	0.30 ± 0.02	0.41 ± 0.02
Oil Stability Index (h)	23.0 ± 0.4	10.1 ± 0.2	3.2 ± 0.1	9.8 ± 0.2
Tocopherols (mg/kg)				
Total	694 ± 8	654 ± 9	720 ± 12	668 ± 5
Alpha	625 ± 7	198 ± 7	229 ± 6	486 ± 5
Beta	37 ± 1	29 ± 1	42 ± 1	30 ± 1
Gamma	22 ± 1	369 ± 8	325 ± 8	131 ± 4
Delta	10 ± 1	59 ± 2	124 ± 2	41 ± 3

Data are expressed as Mean ± SD, $n = 3$.

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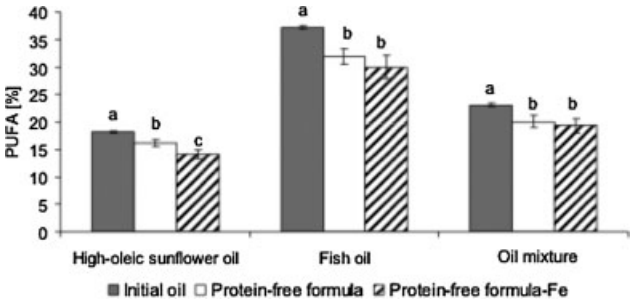

Figure 1. PUFA percentage of initial oils and oils extracted from protein-free formulas (formulas without protein and Tween-20 added) and protein-free formulas-Fe (protein-free formulas containing iron/ascorbate). Data are expressed as Mean ± SD, $n = 3$. Values with different letters indicate significant differences between samples prepared with the same oil.

ascorbate to reduce ferric ions to ferrous ions at a specific concentration. However, this behavior in a food emulsion depends on numerous factors such as pH, temperature, presence of other compounds, oil-droplet interface area, thickness and permeability of the interfacial area, oil concentration, surfactant ionic charge, and especially the molar ratio between ascorbate and the iron [22, 33, 34]

Table 3 shows the results obtained for non-volatile oxidation compounds (total oxidation compounds and its distribution) in oils extracted from formulas including values for oils used for preparation (initial oils). Peroxide values were also determined because it is the method mostly used to evaluate oxidation. In consistency with PUFA loss (Fig. 1), the most oxidized samples were the protein-free formulas, which reached very high contents of oxidation compounds and peroxide values. In fact, all the free-protein samples added the iron/ascorbate combination contained even TAG oligomers, i.e. advanced oxidation compounds.

Table 3. Oxidation levels in initial oils and oils extracted from model formulas

Samples	Oxidation compounds (% of extracted oil)				Peroxide value (meq O_2/kg)
	oxTGM	TGD	TGO	Total	
High-oleic sunflower oil					
Initial oil	1.2	0.3		1.5 ± 0.1^{a}	5.2 ± 0.1^{a}
Protein-free formula	8.3	1.6		9.9 ± 0.9^{b}	146 ± 6^{b}
Protein-free formula-Fe	11.3	2.8	0.9	15.0 ± 1.9^{c}	212 ± 15^{c}
Infant formula	1.5	0.4		1.9 ± 0.3^{a}	8.8 ± 2.1^{a}
Infant formula-Fe	1.5	0.6		2.1 ± 0.3^{a}	6.9 ± 1.9^{a}
Follow-on formula	1.3	0.3		1.6 ± 0.3^{a}	5.3 ± 1.9^{a}
Follow-on formula-Fe	1.4	0.4		1.8 ± 0.3^{a}	6.1 ± 3.1^{a}
Fish oil					
Initial oil	1.7	0.3		2.0 ± 0.1^{a}	9.2 ± 0.2^{a}
Protein-free formula	4.9	3.6	4.4	12.9 ± 2.6^{b}	92 ± 14^{b}
Protein-free formula-Fe	7.8	4.6	5.2	17.6 ± 3.4^{b}	120 ± 17^{b}
Infant formula	3.0	1.1		4.1 ± 0.3^{c}	60 ± 4^{c}
Infant formula-Fe	3.8	1.2	0.4	5.4 ± 0.6^{d}	67 ± 4^{c}
Follow-on formula	1.9	0.4		2.5 ± 0.4^{a}	13 ± 3^{a}
Follow-on formula-Fe	2.0	0.8		2.8 ± 0.3^{a}	17 ± 4^{a}
Mixture oil					
Initial oil	1.5	0.4		1.9 ± 0.1^{a}	6.1 ± 0.1^{a}
Protein-free formula	9.7	2.0		11.7 ± 2.4^{b}	173 ± 18^{b}
Protein-free formula-Fe	13.5	3.1	1.3	16.2 ± 3.7^{c}	260 ± 20^{c}
Infant formula	1.7	0.8		2.5 ± 0.4^{a}	9.5 ± 3.1^{a}
Infant formula-Fe	1.6	0.6		2.2 ± 0.3^{a}	9.2 ± 2.1^{a}
Follow-on formula	1.3	0.7		2.0 ± 0.3^{a}	7.1 ± 2.6^{a}
Follow-on formula-Fe	1.5	0.6		2.1 ± 0.4^{a}	6.2 ± 2.8^{a}

oxTGM, oxidized triacylglycerol monomers; TGD, triacylglycerol dimers; TGO, triacylglycerol oligomers.
Protein-free formula: formula without protein (Tween-20 added); Infant formula: formula with 1:4 sodium caseinate-to whey protein ratio; Follow-on formula: formula with 4:1 sodium caseinate-to whey protein ratio; Protein-free, infant and follow-on formulas-Fe: formulas containing iron/ascorbate.
Data are expressed as Mean ± SD, $n = 3$.
Different letters in each group of samples prepared with the same oil indicate significant differences ($p < 0.05$).

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Regarding the infant and follow-on formulas, significant differences were only found in those containing fish oil, the former showing higher peroxide values and total oxidation compounds. It is important to remark that the percentage of oxidation compounds in the infant and follow-on formulas containing high oleic sunflower oil or the oil mixture simulating the composition of manufactured formulas was low and not significantly different from the initial refined oils.

Concerning comparison of data obtained by the methods used, there were great differences between peroxide values and total oxidation compounds for some samples. According to peroxide values obtained for the free-protein samples, the fish oil samples were less oxidized than the others while the opposite may be deduced from the values of total oxidation compounds. In fact, this apparent contradiction illustrates the advantages of the methodology used to quantitate oxidation compounds over the peroxide value determination, since the latter only provides a partial measure of oxidation (hydroperoxide formation). Since the fish oil samples contained mainly dimers and oligomers, the peroxide value did not reflect the real oxidation level. The level of oxidized TAG monomers has been closely related to peroxide values in the initial stages of oxidation and an excellent linear correlation has been found between both measurements throughout the induction period [35].

Figure 2 shows tocopherol concentrations in the oils extracted from the formulas. Results obtained were consistent with the oxidation levels found (Table 3). The greatest decrease in tocopherol concentration compared to the initial oil corresponded to the samples containing fish oil. Marked losses (45–65%) were found for the protein-free formulas and those with Fe showed significantly lower tocopherol concentration as compared to the ones without it. In addition, significantly lower concentrations of tocopherol were found in the infant versus the follow-on formulas. Given that the protein percentage in both types of formulas was the same (2.5%) but caseinate concentration was lower in the infant formulas, results suggest that caseinate had a greater protective effect than whey proteins in liquid formulas, as already reported [9, 32, 36].

In the case of the formulas simulating the lipid composition of manufactured products, the significant decrease in tocopherols concentration observed following preparation of infant formulas reflects the need to take precautions regarding storage conditions to avoid further losses. Other studies have also shown that tocopherol losses in infant formulas may exceed 50% during storage at RT [18, 36, 37]. In some cases, this could involve that formulas do not reach the minimum levels of vitamin E required by the corresponding regulations upon consumption and, even more, potentially toxic oxidation compounds are likely to be formed.

4 Conclusions

Among all methods used to evaluate oxidation, quantitation of oxidation compounds by SPE and HPSEC provided the most complete information on the oxidation state. It enabled to get to know both primary and secondary oxidation compounds with an only sample analysis instead of applying two or more complementary methods. It is a direct method, being therefore much more sensitive than the PUFA loss determination, and unlike the peroxide value it provides overall information on the degree of oxidation. As to tocopherol determination, it has proved to be a complementary and useful measurement.

The heat treatment used for sterilization did not lead to a significant change in oxidation compounds in infant or follow-on formulas simulating the composition of manufactured products, however the tocopherol concentration decreased significantly. Results obtained for tocopherol losses indicated that the main protective agent against lipid oxidation in model formulas was the protein fraction, especially sodium

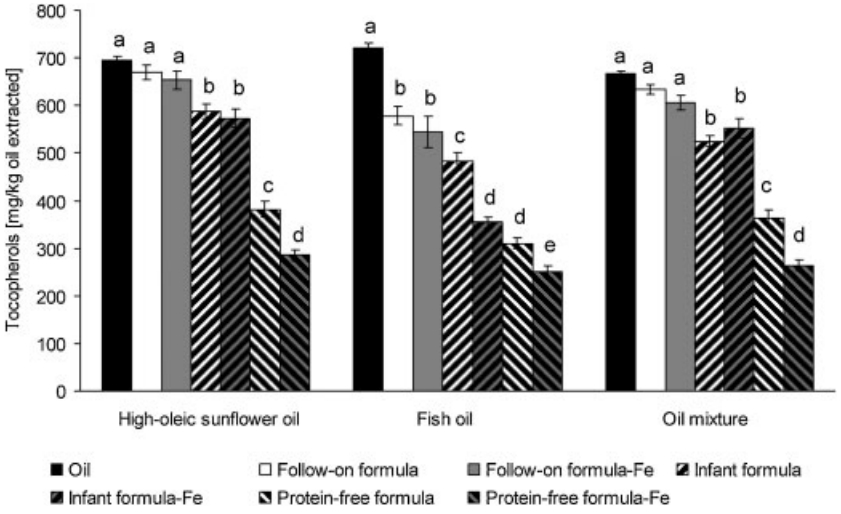

Figure 2. Tocopherol concentration of oils extracted from model formulas. Protein-free formula: formula without protein (Tween-20 added); Infant formula: formula with 1:4 sodium caseinate-to whey protein ratio; Follow-on formula: formula with 4:1 sodium caseinate-to whey protein ratio; Protein-free, infant and follow-on formulas-Fe: formulas containing iron/ascorbate. Data are expressed as Mean ± SD, $n = 3$. Values with different letters indicate significant differences between samples prepared with the same oil.

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caseinate, being more effective a 4:1 than a 1:4 sodium caseinate-to-whey protein ratio. The addition of iron/ascorbate resulted in a prooxidant effect, statistically significant in tocopherol loss but not in formation of oxidation compounds. Overall, this study shows that important losses of tocopherols may occur during formula processing. Given that the shelf-life of infant and follow-on formulas varies from 3 months to as long as 3 years depending on the type of heat treatment applied, it is essential to ensure that no additional antioxidant losses occur during storage and commercialization.

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3.4.- Efecto del tratamiento de esterilización en fórmulas líquidas infantiles embotelladas: impacto sobre la oxidación lipídica y el contenido de tocoferoles.

Effect of sterilization of bottled infant milk: impact on lipid oxidation and tocopherols.

Gloria Márquez-Ruiz, María del Carmen García-Martínez y Francisca Holgado.

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Resumen:

La leche infantil embotellada es susceptible a la oxidación lipídica debido a que su compleja composición incluye ácidos grasos poliinsaturados y minerales prooxidantes como el hierro, que catalizan su oxidación. La mayoría de leches de fórmula son fabricadas a partir de leche de vaca modificada, a la que se retira la fracción grasa y se sustituye por aceites vegetales. En este trabajo se describen las principales variables involucradas en la oxidación de la leche infantil embotellada, así como las interacciones entre sus componentes, destacando la catálisis de los metales y la acción antioxidante. Dichas variables son las que normalmente tienen mayor influencia en los procesos de oxidación en las emulsiones de aceite en agua como la leche. Existen pocos estudios sobre la oxidación lipídica enfocados al efecto de la esterilización y al impacto sobre los tocoferoles, pero los datos obtenidos permiten concluir que el tratamiento térmico utilizado para la esterilización no conduce a una oxidación lipídica significativa aun cuando la concentración de tocoferoles pueda disminuir significativamente; y que entre las múltiples variables involucradas en la oxidación lipídica de la leche infantil embotellada, uno de los principales agentes protectores frente a la oxidación lipídica es la fracción proteica, especialmente el caseinato de sodio. En general, los estudios relacionados con la acción de los tocoferoles en la leche infantil embotellada concluyen que es esencial asegurar niveles óptimos en estos antioxidantes para compensar las pérdidas que ocurren durante el proceso de esterilización y el posterior almacenamiento.

14. Effect of sterilization of bottled infant milk: impact on lipid oxidation and tocopherols

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Abstract

Bottled infant milk is susceptible to lipid oxidation because its complex composition includes polyunsaturated fatty acids and prooxidant minerals such as iron which catalyses their oxidation. Most milk-based formulas are made from bovine milk that has been modified by butterfat removal and vegetable oil addition. In this chapter, the main variables involved in oxidation of bottled infant milk are described, interactions between components, metal catalysis and antioxidant action standing out. Such variables are mostly common to those influencing oxidation in complex oil-in-water milk-like emulsions. Few studies have been found on lipid oxidation in connection with the effect of sterilization and impact on tocopherols but from the data obtained it is concluded that (a) the heat treatment used for sterilization does not lead to significant lipid oxidation even though the tocopherol concentration may decrease significantly and (b) among the multiple variables involved in lipid oxidation of bottle infant milk, one of the main protective agents against lipid oxidation is the protein fraction, especially sodium caseinate. Overall studies related to the action of tocopherols in bottled infant milk conclude that it is essential to ensure optimal levels in these products to compensate for losses occurring during sterilization and further storage.

Keywords: bottled infant milk, lipid oxidation, sterilization, tocopherols, antioxidants, ultra high temperature

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Summary points

- Bottled infant milk is susceptible to lipid oxidation because of its high content in polyunsaturated fatty acids and prooxidant minerals.
- Many variables are involved in lipid oxidation of milk-like emulsions such as bottled infant milk, interactions between components, properties of emulsifiers, metal catalysis and antioxidants action standing out.
- Tocopherols are the most abundant antioxidants, either naturally present or added, in bottled infant milk.
- Sterilization may lead to significant lipid oxidation and tocopherol losses.
- Milk proteins protect against lipid oxidation in bottled infant milk, especially caseinates.

Abbreviations

PUFA	Polyunsaturated fatty acid
UHT	Ultra high temperature

14.1 Introduction

Bottled infant milk is one of the food products most relevant regarding lipid oxidation because the joint addition of prooxidants (minerals such as iron) and oils with a high content of nutritionally important but oxidizable PUFAs such as long-chain omega-3 PUFA, makes it particularly susceptible to oxidative modifications (Joeckel and Phillips, 2009; Nasirpour *et al.*, 2006; Velasco *et al.*, 2008). The inhibition of autoxidation by antioxidants is of the utmost importance. Minor components naturally present in the oils included in formulations, such as tocopherols and polyphenols, exert an essential protective role against lipid oxidation. Lipid oxidation and the effect of antioxidants in infant formulas have been poorly studied. In this context, published research has focused on oxidative modifications of powdered infant formulas occurring during storage, usually evaluated by classic indirect methods (Chávez-Servín *et al.,* 2009; Manglano *et al.,* 2005; Romeu-Nadal *et al.*, 2007) or by the quantitation of residual tocopherols (Albalá-Hurtado *et al.*, 2000a,b; Manglano *et al.*, 2005; Miquel *et al.,* 2004). Classic sterilization is one of the thermal processes most commonly used in the manufacture of bottled infant milk. It can be achieved by applying the proper combinations of heat and time to guarantee microbiological safety but the high temperatures involved could induce oxidation reactions. However, regarding the influence of sterilization treatments on lipid oxidation of bottled milk very scarce work has been found. This chapter summarizes the current knowledge about lipid oxidation in bottled (liquid) infant milk in connection with the effect of sterilization and impact on tocopherols.

14.2 Lipid oxidation and tocopherols: a basic overview

Lipid oxidation during food processing and storage leads to the development of odors and flavors that are responsible for rancidity and greatly influence consumer acceptance (Frankel, 2005). The diverse final distribution of oxidation products depends on many variables that determine the relative speed of reactions between radicals and the subsequent oxidation of the low stability compounds that are formed. Among the variables that may exert an important influence upon the development of oxidation, temperature, oxygen availability, the presence of antioxidants and prooxidants and the degree of unsaturation of fatty acids are prominent. The complexity of oxidation greatly increases in emulsions such as bottled milk because of the effect of additional variables such as the discontinuity of the lipid phase, homogenization conditions, pH and fat globule size, among others (Let *et al.,* 2007a; Velasco *et al.,* 2002; Waraho *et al.*, 2010).

Tocopherols are by far the most abundant antioxidants found in milk and infant formula, either naturally present or added. They are phenolic antioxidants (AH) that act as chain-breaking

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antioxidants by different mechanisms being the main one the interruption of the propagation chain of autoxidation by reaction with peroxyl radicals (LOO•) to produce less reactive species (Frankel, 2007). The phenoxyl radicals (A•) produced are stabilized by resonance. Also, tocopherols can react with the two other main radicals involved: alcoxyl radicals (LO•) and free radicals (L•), as described below:

LOO• + AH → LOOH + A•
LO• + AH → LOH + A•
L• + AH → LH + A•

The phenoxyl radicals (A•) can further contribute to formation of stable products, including partially oxidized structures which keep antioxidant activity *via* the same hydrogen donor property of the parent compound:

A• + A• → A – A
LO• + A• → LOA
LOO• + A• → LOOA

However, other reactions with radicals can occur, ultimately giving rise to stable, oligomeric structures:

LOO• + AH → LOOAH•
LOOAH• + LOO• → LOOAHOOL

At high concentrations tocopherols may act as prooxidants by initiating free radical formation (*Mukai et al.*, 1993). Tocopherols activity greatly depends on temperature, lipid composition and physical state in multiphase systems. At low or moderate temperatures, tocopherol loss is more rapid ##as unsaturation degree is higher (Kamal-Eldin and Appleqvist, 1996) as well as it is the oxidation level achieved upon exhaustion of tocopherols## (Martín-Polvillo *et al.*, 1996) while the opposite seems to occur at high temperatures (Jorge *et al.*, 1996).

14.3 Lipid oxidation and antioxidants in milk-like emulsions

Lipid oxidation in systems in which the fat or oil is dispersed as emulsion droplets such as bottled milk is still poorly understood in spite of the large number of foods that consist either wholly or partially of emulsions, or that have been in an emulsified form at some time during their production (Coupland and McClements, 1996; Skibsted *et al.*, 2010; Velasco *et al.*, 2002; Waraho *et al.*, 2010). In milk-like emulsions, lipid droplets are dispersed in a continuous water phase, stabilized by proteins, phospholipids or surfactants. Particularly susceptible to oxidation are milk products enriched with omega-3 PUFAs or conjugated linoleic acid to improve their beneficial health effects (García-Martínez and Márquez-Ruiz, 2009).

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A number of variables besides those generally involved in lipid oxidation (temperature, oxygen availability and the degree of unsaturation of fatty acids) have a prominent influence on lipid oxidation and the effect of antioxidants in milk-like emulsions:

- Properties (composition, location, concentration) and modifications (changes in electric charge, permeability, and others) of the emulsifier that constitutes the interface. The emulsifier can provide a protective barrier to the penetration and diffusion of radicals or molecules that initiate lipid oxidation (Frankel, 2005). Electric charge of ionic emulsifiers influences greatly the oxidation rate. Higher concentrations of emulsifier can increase oxidative stability by causing tighter and thicker packing at the interface, hence becoming a more efficient barrier against diffusion of lipid oxidation initiators.
- Partition of reactants and products between the interfacial region, oil and water phases due to their polarity and surface activity. Lesser polar molecules are mainly located in the oil phase, polar molecules in the water phase and amphiphilic molecules in the interface. Orientation of molecules in this interfacial region affects the accessibility to oxygen of reactive species as peroxides, hydroxyl and perhydroxyl radicals which are soluble in water (Coupland and McClements, 1996). The partition of scission products between the oil and water phases can have an important influence on sensorial perception in milk-like emulsions as aromatic compounds are perceived more intensely in the water phase (Sims, 1994).
- Interaction of lipids with other components of the milk-like emulsions which can act as prooxidants or antioxidants depending on their chemical properties. Carbohydrates can either protect against lipid oxidation by joining with metals and radicals (Yamauchi *et al.*, 1982) or accelerate oxidation (Yamauchi *et al.*, 1988). The effectiveness of proteins to delay the oxidation in emulsions depends on their conformation, interface composition and partition between the interface and the water phase. Besides trapping free radicals, their antioxidant effect has been attributed to their capacity for formation of viscoelastic dense membranes that restrict the penetration or diffusion of initiators of oxidation inside the droplets (Tong *et al.*, 2000). Milk-like emulsions are protected from lipid oxidation by proteins like casein capable of binding and inactivating copper and iron as well as protecting the oil-water interface around the fat globule membrane. Proteins containing reducing sulfhydryl groups are particularly effective as antioxidants in milk, for this reason oxidative stability is increased when milk is heated resulting in release of free sulfhydryl groups of proteins.
- Effect of pH: lipid oxidation rate is in general lower at high pHs and increases as the pH decreases, which may be attributed to solubilization of metal catalysts.
- Metal catalysis: hydrophilic metals are located towards the interface and initiation of oxidation occurs in the lipid phase inside the interface (Schaich, 1992). Therefore, chelating agents and reaction conditions that increase metal concentration in this site enhance their effectiveness. Charge of chelators and electrostatic environment that surround metals can affect their redox potential and other thermodynamic properties. Metal catalysts are recognized as the most important factors in accelerating lipid oxidation in milk-like emulsions. Although copper occurs naturally in milk at lower concentration (20-40 µg/l) than iron (100-250 µg/l), it is the most important catalyst in the development of oxidized flavors (Juhlin *et al.*, 2010). Metal chelators such as EDTA reduce the development of oxidized flavors but the use of these additives is not allowed in some countries. Iron supplementation to meet the nutritional

requirements of infant milks can seriously limit the shelf-life of these products. Lactoferrin (a non-heme iron-binding glycoprotein found in human milk much more abundantly and less saturated with iron than in bovine milk) has been proposed as an antioxidant additive in infant formulas (Frankel, 2005).

Antioxidants in multiphase systems like milk-like emulsions are distributed between the water phase, the oil phase and the interfacial region, rich in emulsifier, depending on their affinity and relative amounts. Lipophilic antioxidants, such as tocopherols, are more effective in oil-in-water emulsions than are hydrophilic antioxidants, whereas the contrary effect occurs in bulk oils, which is known as the 'polar paradox'. In oil-in-water emulsions, lipophilic antioxidants tend to be orientated in the oil-water interface and hence protect oil against oxidation, while hydrophilic antioxidants tend to dissolve and become diluted in the water phase (Frankel, 2007; Porter, 1980).

Alpha-tocopherol is the only important natural antioxidant in milk, which contains an average of 25 μg/g milkfat and 44 μg/g fat globule membrane and the oxidative stability of milk correlates well with the alpha-tocopherol level (Frankel, 2005).

In bottled infant milk, alpha-tocopherol concentration may decrease significantly over time during bottle feeding thus indicating it is essential to ensure sufficient tocopherol levels in these products (Francis *et al.*, 2012). Alpha-tocopherol has shown a clear protective action both on radical formation and peroxide development on early events in lipid oxidation in milk-like emulsions and hence supplementation in bovine diets or by direct addition in products is recommended (Skibsted *et al.,* 2005). In turn, ascorbic acid acts as a synergist of tocopherols in milk at normally low copper concentrations but its content decreases continuously during storage and is depleted by consuming dissolved oxygen (Frankel, 2005). The loss of tocopherols is closely related with the degree of unsaturation of the oil or lipids involved. Hence contents of tocopherols decreased significantly during storage in milks enriched with fish oil (Let *et al.*, 2007b). Many studies have been carried out in this context by Jacobsen *et al.* directed to study the effects of different antioxidants, homogenization conditions and protein composition on lipid oxidation of milk emulsions (Jacobsen *et al.,* 2008; Let *et al.*, 2003, 2004, 2005, 2007a,b; Sorensen *et al.,* 2007).

14.4 Lipid oxidation and sterilization in milk

The principal objective for milk and milk-like emulsions preservation is inactivation of microorganisms and enzymes by applying elevated temperatures for a short time, while simultaneously protecting nutritional and sensory properties. In UHT treatment, milk is heated at 130-140 °C for 3-5 seconds leading to sterilized products with shelf-life extended to a few months when stored at ambient temperature. UHT milk is more susceptible to metal or light-induced oxidation because ascorbic acid is depleted early in the shelf-life of the product, especially at warmer (>7 °C) temperatures (Duncan and Webster, 2010). Consequently, there is a great interest on development of more protective packaging materials (Mortensen, 2010). Flexible monolayer low-density polyethylene clear or pigmented with titanium oxide, and multiple-layer

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coextruded low-density polyethylene/polyamide pigmented with carbon black and titanium oxide are suggested as new flexible packaging materials to effectively extend the shelf-life of milk-like emulsions including bottled infant milk.

Oxidized flavours in milk and milk-like emulsions such as bottled infant milk formulas are detected and easily perceived at extremely low levels (parts per billion) because the low molecular weight off-flavour compounds are more readily volatilized in the predominantly aqueous matrix of these products. Alkanals, 2-alkenals and 2,4-alkadienals with more than five carbons and ketones are typical volatiles produced (Frankel, 2005). Evolution of volatile components in UHT milks during storage have been well studied (Contarini *et al.,* 1997; Rerkrai *et al.*, 1987; Valero *et al.,* 2001) including analysis of aldehydes originated from oxidation of unsaturated fatty acids. However, the influence of UHT treatment on formation of volatile oxidation products is not clear.

Herzallah *et al.* (2005) have found that UHT caused a significant although very low decrease of the conjugated linoleic acid content of milks while the treatment did not influence on *trans* isomer formation. Focus on changes of conjugated linoleic acid and *trans* isomers is supported by the enormous current interest in keeping the former intact because of its potential anticarcinogenic effects and avoiding formation of the latter because of their deleterious implications on lipid metabolism and cardiovascular diseases.

As to formation of cholesterol oxidation products, of great concern because of their cytotoxic, atherogenic and carcinogenic effects (Vicente *et al.,* 2012), results obtained are inconclusive. Some studies suggest that cholesterol oxidation products are not formed by UHT treatment and can be found only in processed dairy products exposed to harsh storage conditions where impact of oxygen and light or oxygen and low water activity are concomitant (Sieber, 2005) while others show the opposite (Calderon-Santiago *et al.*, 2012; Pikul *et al.*, 2013).

In the recent publication of Pikul *et al.* (2013) degradation of cholesterol and formation of cholesterol oxidation products were observed in UHT milk during thermal treatment and storage. To illustrate the results obtained, some data have been shown in Figure 14.1 including formation of oxysterols in bovine milk after UHT treatment and during storage at 4 °C and 20 °C for 2, 4 and 6 months. After UHT treatment, the amounts of these compounds increased by 58% and, irrespective of storage temperature, continuous increases in the total amounts of cholesterol oxidation products were observed over storage. UHT treatment initiated oxidation at the side chain of cholesterol, forming 20% of 25-hydroxycholesterol. After 6 months of storage, 7-hydroxycholesterol epimers dominated among oxysterols.

14.5 Specific studies on lipid oxidation and impact on tocopherols in sterilized bottled infant milk

Bottled infant milk is highly susceptible to lipid oxidation because its complex composition includes polyunsaturated fatty acids and prooxidant minerals such as iron that catalyze their

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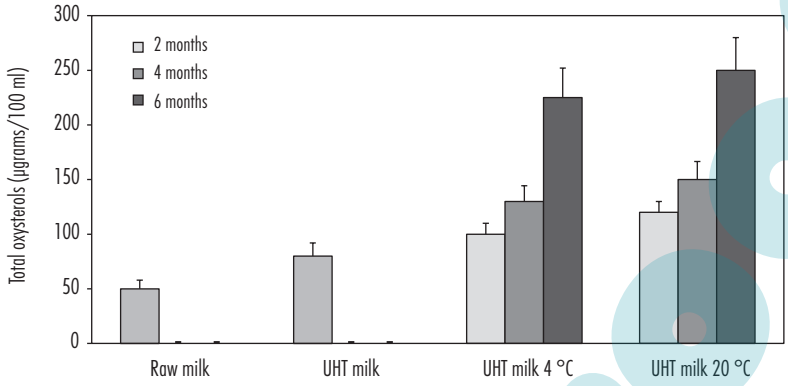

Figure 14.1. Oxysterols in raw milk, ultra high temperature (UHT) milk and UHT milk during storage. This figure depicts levels of oxysterols (µgrams/ml) in raw milk samples (mean±SD, n=3) and their increase following UHT treatment. It also includes changes of oxysterols in UHT milk samples (mean±SD, n=3), during storage at 4 °C and 20 °C for 2,4 and 6 months (Pikul *et al.*)

oxidation. Most milk-based formulas are made from bovine milk that has been modified by butterfat removal and vegetable oil addition. To improve the taste and nutritional quality, more carbohydrates are often added, usually lactose, and some proteins are removed. Various bovine milk-derived proteins (e.g. non-fat milk, casein, combinations of casein and whey proteins or partially hydrolyzed whey protein concentrate) are often used for these formulas. Carbohydrates are normally provided by lactose, corn syrup solids, sucrose or corn maltodextrins. Casein-predominant formulas consist of dilute bovine skim milk to which fat and other nutrients are added but the casein:whey ratio is not changed, while whey contents are increased in whey-predominant formulas to get closer to the whey:casein ratio in human milk. For infants with allergies or intolerance to milk proteins or lactose, soy protein-, protein hydrolysate- and amino acid- based formulas are available. Regarding the oils used, the main ones are coconut, soybean, high-oleic sunflower, low-erucic acid rapeseed, palm, palm kernel and peanut oils, normally supplemented with different sources of eicosapentaenoic acid and docosahexaenoic acid, such as fish oils, egg lipids or phospholipids and oils derived from microalgal and fungal sources (Nasirpour *et al.*, 2006).

Few studies have been found on lipid oxidation in bottled (liquid) infant milk in connection with the effect of sterilization and impact on tocopherols and results obtained are commented below.

In studies directed to evaluate the stability of vitamins during the storage of sterilized liquid infant milks, Albalá-Hurtado *et al.* (2000a,b) examined changes of tocopherols (vitamin E) at 20, 30 and 37 °C. Samples were commercial follow-on formulas which had been conventionally sterilized (15 min at 115 °C) and canned in crown-capped brown glass. Table 14.1 shows the initial and final concentrations of vitamin E after 12 months-storage at the temperatures tested. It is important to note that the initial content of vitamin E was much higher than the minimum generally recommended. As can be observed, no changes were found in vitamin E levels throughout

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Table 14.1. Vitamin E contents (mg/100 ml) in follow-on liquid milks. This table shows levels of Vitamin E (mg/100 ml) (mean±SD, n=2) in samples of follow-on liquid formulas before (initial) and after 12-months storage at 20 °C, 30 °C and 37 °C (Albalá-Hurtado *et al.*, 2000).

	Initial	12 months at 20 °C	12 months at 30 °C	12 months at 37 °C
Vitamin E (mg/100 ml)	0.99±0.08	0.98±0.07	0.92±0.06	1.06±0.08

storage not even at 37 °C. In contrast, vitamin A showed a slight decrease at 20 °C and 30 °C and significant losses at 37 °C (data not shown).

The objective of our recent study in this context was to evaluate the effect of classic sterilization on lipid oxidation of liquid infant and follow-on formulas by analysing formation of non-volatile oxidation compounds, including oxidized and dimeric triacylglycerols (García-Martínez *et al.*, 2012). Model systems containing similar components and proportions to those normally found in manufactured samples and a mixture of high-oleic sunflower oil, rapeseed oil and fish oil were used to obtain a fatty acid composition profile in accordance with the EU regulations. For comparative purposes, some samples were prepared with high-oleic sunflower or fish oil and others without the protein components and added Tween-20 (Velasco *et al.*, 2004). Quantification of total oxidized triacylglycerols provided complete information of the oxidation state and showed clear advantages vs. other methods normally used, i.e. loss of unsaturated fatty acids and peroxide value.

Table 14.2 shows the results for peroxide values, total oxidation compounds and oil stability index in the oils used for preparation of infant and follow-on formulas. Peroxide value data were those expected for refined oils (below 10 meq O_2/kg oil). The fish oil contained the highest level of peroxides, being the most susceptible to oxidation among the oils used due to its high PUFA content, specifically docosahexaenoic acid (13.0%). Total oxidation compounds were analyzed following a methodology based on combination of chromatographic techniques (Márquez-Ruiz and Dobarganes, 2005; Márquez-Ruiz *et al.*, 1996). The results obtained showed oxidation levels commonly found in refined oils. With respect of oil stability index, the fish oil had a value of 3.2 hours, while the high oleic sunflower oil was the most stable, with an oil stability index of 23.0 hours and both the low erucic rapeseed oil and the mixture oil prepared showed intermediate values.

The total tocopherol contents (Table 14.3) were similar for the three oils, whereas their distribution in different tocopherol types was quite different. The fish oil had a slightly higher total tocopherol concentration, being remarkable its content of gamma- and alpha-tocopherol. In the case of high oleic sunflower oil, the tocopherols essentially consisted of alpha, while the low erucic rapeseed oil had the highest and lowest concentrations of gamma- and alpha-tocopherol, respectively.

Early proof

Table 14.2. Oxidation parameters of the oils used in the preparation of model liquid infant formulas. This table shows levels of peroxide value (meq O_2/kg oil), total oxidation compounds (% on oil) and oil stability index (h) (mean±SD, n=3) in the oils used in the preparation of model liquid infant formulas (García-Martínez *et al.*, 2012).

	High-oleic sunflower oil	Rapeseed oil	Fish oil	Mixture oil
Peroxide value (meq O_2/kg)	5.2±0.1	7.8±0.5	9.2±0.2	6.1±0.1
Total oxidation compounds (%)	1.5±0.1	2.5±0.1	2.0±0.1	1.9±0.1
Oil stability index (h)	23.0±0.4	10.1±0.2	3.2±0.1	9.8±0.2

Table 14.3. Tocopherol contents of the oils used in the preparation of model liquid infant formulas. This table shows levels (mg/kg) (mean±SD, n=3) of total tocopherols and their distribution in alpha-, beta-, gamma- and delta- tocopherols (García-Martínez *et al.*, 2012).

Tocopherols (mg/kg)	High-oleic sunflower oil	Rapeseed oil	Fish oil	Mixture oil
Total	694±8	654±9	720±12	668±5
Alpha	625±7	198±7	229±6	486±5
Beta	37±1	29±1	42±1	30±1
Gamma	22±1	369±8	325±8	131±4
Delta	10±1	59±2	124±2	41±3

Table 14.4 shows the results obtained for total oxidation compounds and peroxide values in oils extracted from formulas as compared with values for oils used for their preparation (initial oils). The most oxidized samples were the protein-free formulas, which reached very high contents of oxidation compounds and peroxide values.

These results indicated, firstly, that milk proteins exerted an important protective action on lipids in terms of oxidation. Multiple pathways may be involved in such effects, including inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties of food systems (Villiere *et al.*, 2005). Furthermore, it has been recently reported that unadsorbed proteins exert greater antioxidative effects than do adsorbed proteins (Berton *et al.*, 2011). In the particular case of casein, the ability of phosphorylated groups to chelate pro-oxidant metals ions may explain this inhibition, but free-radical-scavenging activity could also be involved (Elias *et al.*, 2008).

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Table 14.4. Oxidation levels in oils extracted from model liquid formulas. This table shows total oxidation compounds (% of extracted oil) (mean±SD, n=3) and peroxide values (meq O_2/kg extracted oil) (mean±SD, n=3) in model liquid infant formulas. Protein-free formula: formula without protein (Tween-20 added); infant formula: formula with 1:4 sodium caseinate-to whey protein ratio; follow-on formula: formula with 4:1 sodium caseinate-to whey protein ratio; protein-free, infant and follow-on formulas-Fe: formulas containing iron/ascorbate (García-Martínez *et al.*, 2012).

Samples		Total oxidation compounds (% of extracted oil)[1]	Peroxide value (meq O_2/kg)
High-oleic sunflower oil	Initial oil	1.5±0.1^a	5.2±0.1^a
	Protein-free formula	9.9±0.9^b	146±6^b
	Protein-free formula-Fe	15.0±1.9^c	212±15^c
	Infant formula	1.9±0.3^a	8.8±2.1^a
	Infant formula-Fe	2.1±0.3^a	6.9±1.9^a
	Follow-on formula	1.6±0.3^a	5.3±1.9^a
	Follow-on formula-Fe	1.8±0.3^a	6.1±3.1^a
Fish oil	Initial oil	2.0±0.1^a	9.2±0.2^a
	Protein-free formula	12.9±2.6^b	92±14^b
	Protein-free formula-Fe	17.6±3.4^b	120±17^b
	Infant formula	4.1±0.3^c	60±4^c
	Infant formula-Fe	5.4±0.6^d	67±4^c
	Follow-on formula	2.5±0.4^a	13±3^a
	Follow-on formula-Fe	2.8±0.3^a	17±4^a
Mixture oil	Initial oil	1.9±0.1^a	6.1±0.1^a
	Protein-free formula	11.7±2.4^b	173±18^b
	Protein-free formula-Fe	16.2±3.7^c	260±20^c
	Infant formula	2.5±0.4^a	9.5±3.1^a
	Infant formula-Fe	2.2±0.3^a	9.2±2.1^a
	Follow-on formula	2.0±0.3^a	7.1±2.6^a
	Follow-on formula-Fe	2.1±0.4^a	6.2±2.8^a

[1] The different superscript letters within each group of samples prepared with the same oil indicate significant differences ($P<0.05$).

Secondly, the differences between samples with and without Fe were lower than expected, and only significant in the high-oleic sunflower oil samples. The prooxidant effect of the iron/ascorbate complex is based on the capacity of ascorbate to reduce ferric ions to ferrous ions at a specific concentration. However, this behavior in a food emulsion depends on numerous factors such as pH, temperature, presence of other compounds, oil-droplet interface area, thickness and permeability of the interfacial area, oil concentration, surfactant ionic charge, and especially the molar ratio between ascorbate and the iron (Branco *et al.*, 2011; Buettner and Jurkiewicz, 1996).

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Regarding the infant and follow-on formulas, significant differences were only found in those containing fish oil, the former showing higher peroxide values and total oxidation compounds. It is important to remark that the % of oxidation compounds in the infant and follow-on formulas containing high oleic sunflower oil or the oil mixture simulating the composition of manufactured formulas was low and not significantly different from the initial refined oils.

Concerning comparison of data obtained by the methods used, there were great differences between peroxide values and total oxidation compounds for some samples. According to peroxide values obtained for the free-protein samples, the fish oil samples were less oxidized than the others while the opposite may be deduced from the values of total oxidation compounds. In fact, this apparent contradiction illustrates the advantages of the methodology used to quantitate oxidation compounds over the peroxide value determination, since the latter only provides a partial measure of oxidation (hydroperoxide formation). Since the fish oil samples contained mainly dimers and oligomers, the peroxide value did not reflect the real oxidation level.

The significant decrease in tocopherols concentration observed following preparation of infant formulas simulating the lipid composition of manufactured products (Figure 14.2) reflects the need to take precautions regarding storage conditions to avoid further losses. Other studies have also shown that tocopherol losses in infant formulas may exceed 50% during storage at room temperature (García-Martínez *et al.*, 2010; Hu *et al.*, 2003; Miquel *et al.*, 2004). In some cases

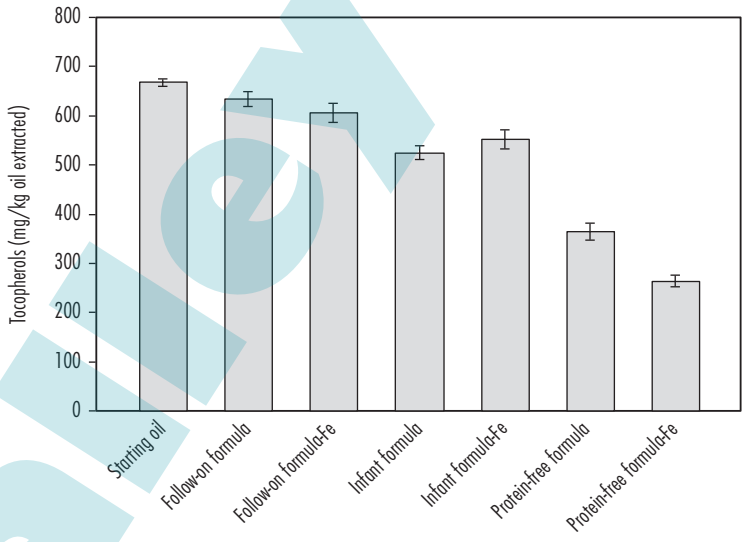

Figure 14.2. Tocopherol concentrations in the oils extracted from model liquid formulas prepared with the mixture oil. This figure depicts levels of total tocopherols (mg/kg oil extracted) (mean±SD, n=3) in the starting oil (mixture oil); protein-free formula (without protein, with Tween-20 added); infant formula (with 1:4 sodium caseinate-to whey protein ratio); follow-on formula (with 4:1 sodium caseinate-to whey protein ratio); protein-free, infant and follow-on formulas-Fe: formulas containing iron/ascorbate (García-Martínez *et al.*, 2012).

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this could involve that formulas do not reach the minimum levels of vitamin E required by the corresponding regulations upon consumption and, even more, potentially toxic oxidation compounds are likely to be formed. Marked losses (45-65%) were found for the protein-free formulas and those with Fe showed significantly lower tocopherol concentration as compared to the ones without it. In addition, significantly lower concentrations of tocopherol were found in the infant vs. the follow-on formulas. Given that the protein % in both types of formulas was the same (2.5%) but caseinate concentration was lower in the infant formulas, results suggest that caseinate had a greater protective effect than whey proteins in liquid formulas, as already reported (Elias *et al.*, 2008; Hu *et al.*, 2003; Waraho *et al.*, 2010).

Results showed that the heat treatment used for sterilization did not lead to significant lipid oxidation, but the tocopherol concentration decreased significantly. The marked tocopherol losses found in protein-free formulas together with the significantly lower tocopherol concentrations in infant formulas (80% whey in protein fraction) compared to follow-on formulas (80% caseinate in protein ratio) showed the protective effect of the protein fraction, especially sodium caseinate. Overall, this study shows that important losses of tocopherols may occur during liquid infant formula sterilization, but such losses depend greatly on a number of variables pertaining to formula compositions (proteins and prooxidants among others).

14.6 Practical guidelines

- Supplementation with tocopherols up to levels that compensate for losses occurring during sterilization and storage may be required in bottled infant milk.
- The application of methods which provide complete information on the oxidized compounds formed at different stages of oxidation is recommended to control quality and ensure safety of bottled infant milk.
- Modifications of milk protein composition in formulations may be required to contribute to increase oxidative stability of bottled infant milk.
- Benefit-risk assessments are required regarding recommendations of supplementation with prooxidant minerals in bottled infant milk.

Acknowledgements

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3.5.- Estabilidad lipídica de fórmulas infantiles en polvo almacenadas a temperatura ambiente.

Lipid stability in powdered infant formula stored at ambient temperatures.

María del Carmen García-Martínez, Luis Miguel Rodríguez-Alcalá, Susana Marmesat, Leocadio Alonso, Javier Fontecha y Gloria Márquez-Ruiz.

International Journal of Food Science and Technology, 2010; 45: 2337-2344.

Resumen:

La estabilidad oxidativa de fórmulas infantiles en polvo se evaluó a temperaturas ambiente (25, 30 y 37ºC) durante 3 meses. La fracción lipídica se analizó exhaustivamente para evaluar los cambios en la composición de ácidos grasos y en los isómeros de ácidos grasos trans, compuestos de oxidación no volátiles incluyendo triglicéridos oxidados monómeros, dímeros y polímeros, y los antioxidantes (tocoferoles). No se encontraron cambios significativos en ninguno de los parámetros examinados en los lípidos totales a lo largo del periodo de almacenamiento. Sin embargo las fracciones minoritarias de aceite libre o superficial (aproximadamente un 7,5% de los lípidos totales) mostraron un incremento significativo de compuestos de oxidación y un marcado descenso en los niveles de tocoferoles durante el almacenamiento a temperatura ambiente. Las muestras almacenadas a 37ºC durante 3 meses mostraron rancidez y consistentemente contenían niveles de oxidación más elevados en la fracción de aceite libre mientras que, en contraste, los lípidos totales extraídos no estaban aparentemente oxidados. Los resultados indican la necesidad de analizar separadamente la fracción de aceite libre o superficial en fórmulas infantiles para obtener una información más completa del estado de oxidación.

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Original article

Lipid stability in powdered infant formula stored at ambient temperatures

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Summary Lipid stability of standard infant formula was evaluated at ambient temperatures, namely 25, 30 and 37 °C, during 3 months. Lipids were thoroughly analysed to evaluate changes in fatty acid composition and trans fatty acid isomers, non-volatile oxidation compounds including oxidised, dimeric and polymeric triacylglycerols, and tocopherol. No significant changes in either of the parameters examined were found in total lipids extracted from infant formula along the storage period. However, the minor free oil fractions (about 7.5% of total lipids) showed a significant increase in oxidation compounds and marked decrease in tocopherol levels during storage at all temperatures. Samples stored at 37 °C for 3 months were rancid and, accordingly, contained the highest oxidation level in the free oil fraction, whereas total lipids extracted were apparently not oxidised. Results showed the necessity of analysing separately the free oil fraction in infant formulae to obtain a clear picture of the oxidation status.

Keywords Free oil, infant formula, oxidation compounds, storage, tocopherol.

Introduction

Powdered infant formulae normally show fatty acid compositions similar to those of human milk, generally achieved by combination of vegetable oils such as sunflower, soybean, coconut, palm, high-oleic safflower, peanut, low-erucic acid rapeseed and corn oils (Nasirpour *et al*., 2006). A number of formulations are nowadays supplemented with long-chain polyunsaturated fatty acids (LC-PUFA) as it has been reported that preterm and possibly new-born infants are not capable of synthesising sufficient amounts of such fatty acids to cover their needs for brain and retina development (Fleith & Clandinin, 2005). Also, vitamin and mineral fortification is necessary to fulfil nutritional requirements of infant formulae. As a consequence, infant formulae normally contain a relatively large amount of unsaturated, oxidisable fatty acids together with non-negligible amounts of prooxidant minerals, including iron, and hence it is essential to control lipid stability during storage to ensure their nutritional value and safety (Nasirpour *et al*., 2006).

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Changes in lipid oxidation parameters during storage of powdered infant formulae have been reported in a few studies (Angulo *et al*., 1998; Chávez-Servín *et al*., 2008a; De La Presa-Owens *et al*., 1995; García-Llatas *et al*., 2006; Manglano *et al*., 2005; Michalski *et al*., 2008; Rodríguez-Alcala *et al*., 2007; Romeu-Nadal *et al*., 2007; Thomkinson & Mathur, 1990; Velasco *et al*., 2008) but the complexity of both the food matrix and the oxidation process usually leads to fluctuations or inconsistencies in oxidation measurements (De La Presa-Owens *et al*., 1995; Angulo *et al*., 1998; Manglano *et al*., 2005; García-Llatas *et al*., 2006; Chávez-Servín *et al*., 2008a). Among the major causes for the confusing data obtained is the influence of the extraction procedures applied on the methods used for the analysis of oxidation, as occurs with peroxide value determination (Velasco *et al*., 2008).

It is poorly known whether the changes in the lipid fraction during storage may occur within temperature ranges close to ambient conditions, including 25 °C as the usual room temperature in markets and food stores, and up to 30 and 37 °C, which can be reached in certain stores and houses during summer. In our previous work, lipid composition of conventional powdered infant formulae was thoroughly analysed during 4 years of

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storage at 25 °C, and no relevant changes were found in PUFA, trans isomers or oxidation compounds (Rodríguez-Alcala *et al*., 2007). However, powdered infant formulae highly supplemented with n-3 LC-PUFA and stored at ambient temperatures showed especially marked changes in PUFA, volatile formation and sensory analysis at 37 °C (Romeu-Nadal *et al*., 2007). Various studies have focused on tocopherol analysis of powdered infant formulae during storage because the stability of tocopherols is essential to guarantee the minimum vitamin E content required by legislations and to protect lipids from oxidation. Tocopherols are the main lipid antioxidants present in infant formulae and are derived from the vegetable oils used as ingredients and from specific addition during their manufacture. Great losses of tocopherols in infant formulae supplemented with iron lactate or sulphate after 17 months of storage at 37 and 22 °C have been detected (Miquel *et al*., 2004). Also, losses of about 13% and 20% after 12 months of storage at 25 and 40 °C, respectively, have been reported (Chávez-Servín *et al*., 2008b). In contrast, no changes were observed in commercially available infant formulae stored at 20, 30 and 37 °C for 12 months (Albala-Hurtado *et al*., 2000). However, none of these studies provide data on the two lipid fractions that can be separately extracted in infant formulae. Most lipids are immersed in the solid matrix and therefore called encapsulated, and their extractions require disruption of the matrix by physical or chemical means. However, there is a minor lipid fraction easily extractable by organic solvents and thus called free, which is more accessible to air and hence could be more susceptible to oxidation. The importance of analysing the free oil fraction in milk powders for its influence on physical properties and stability has been highlighted (Vignolles *et al*., 2007). Still, only in our previous paper, free oil fractions were analysed separately in infant formulae, and this approach allowed to detect that tocopherol levels were substantially lower (10–15%) in the free oil fractions than in the total oils extracted all throughout storage at 25 °C for 4 years (Rodríguez-Alcala *et al*., 2007). Such results supported the interest of investigating the susceptibility of oxidation of the free oil fraction during storage, as already reported for other powdered products (Velasco *et al*., 2006).

In this work, a conventional infant formula was stored at 25, 30 and 37 °C under accelerated oxidation conditions to evaluate lipid stability and oxidation of free oil fractions. Analyses included fatty acid profile, specific determination of conjugated linoleic acid (CLA) isomers and trans fatty acids, quantitation of total non-volatile oxidation compounds including dimeric and oxidised triacylglycerols and quantitation of tocopherol isomers. Oxidation parameters were analysed both in total oils and in the free oil fractions extracted from infant formula.

Materials and methods

Samples

Milk-based adapted infant formula (Baby 2) was provided by a local industry (Hero España, S.A., Murcia, Spain). Composition of the formula is shown in Table 1. Ingredients in decreasing order of quantity were maltodextrins, skimmed milk, vegetable oils, demineralised milk whey, minerals, lactose, lecithin, vitamins (A, C, E, B_1, B_2, B_6, B_{12}, D_3, K, niacin, pantothenic acid and folic acid), choline, taurine, inositol and carnitine. Infant formula was elaborated in the local industry as follows: milk was skimmed, pasteurised (72 °C per 15 s) and concentrated (falling film evaporator at 85, 66 and 58 °C during 5 min). Then, pasteurised whey (72 °C per 15 s), lipids (a blend of vegetable oils), lactose and minerals were added, mixed and sterilised (high-temperature short-time treatment at 100 °C per 22 s). Finally, the rest of the ingredients were added and spray-dried by atomisation (air input 175–185 °C; air output 90–94 °C).

Samples were divided into 200-g batches and stored at 25, 30 or 37 °C in the dark in sealed plastic bags (supplied by Criovac® BB4L; Sealed Air SL, Barcelona, Spain) in air, for 3 months. Samples were analysed each month in triplicate.

Lipid extraction

For analysis of fatty acid methyl esters (FAMEs) and trans fatty acids, lipids were extracted following a procedure described by an International Standard Method for milk powder (ISO, 2001). Briefly, it consists of the addition of an ammoniacal ethanolic solution applied to a test portion followed by lipid extraction using diethyl ether and pentane. Then, the upper layer is removed, and the solvent is completely evaporated.

For the analyses of oxidised compounds and tocopherols, the fraction of free oil was additionally extracted. The free oil fraction, also known as the non-encapsulated oil fraction, was determined (Sankarikutty *et al*., 1988). Thus, 200 mL of light petroleum ether (60–80 °C) was added to 4 g of powder sample. Stirring was applied at room temperature for 15 min. After filtration through a filter paper, the solvent was

Table 1 Composition of infant formula. Data obtained from the company and based on label claims

	Infant formula	
	100 g powder	**100 mL**
Energy (kcal)	500	70
Proteins (g)	15.0	2.1
Carbohydrates (g)	53.7	7.5
Fat (g)	25.0	3.5

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evaporated in a rotary evaporator and the extracted oil was dried to constant weight using a stream of nitrogen.

Preparation of FAME

FAMEs were prepared by base-catalysed methanolysis of the extracted lipids using KOH (2 M) in methanol as described by International Standard ISO-IDF (ISO, 2002).

Silver argentation thin-layer chromatography (Ag^+-TLC)

FAMEs were fractionated according to the number and geometry of double bounds by TLC. Briefly, the TLC glass plates (Merck, Darmstadt, Germany) were incubated with 20% aqueous solution of silver nitrate (Panreac, Barcelona, Spain) for 16 h, partially air-dried and activated at 120 °C for 30 min. A 45-μL solution of FAME (100 mg mL^{-1}) was applied in a narrow band, and the plates were developed twice in a saturated chamber containing hexane and diethyl ether (9:1, v/v). The plates were air-dried and sprayed with a 0.20% ethanol solution of 2′,7′dicloroflurescein (Merck), and the bands were visualised under UV light. The bands were scrapped off and the compounds eluted with diethyl ether and then analysed by GC as described below.

GC-FID analysis

FAMEs were analysed on a Perkin-Elmer chromatograph (Autosystem, Beaconsfield, UK) with a FID detector. A CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness; Chrompack, Middelburg, The Netherlands) was used. The column was held at 100 °C for 1 min after injection, temperature-programmed at 7 °C min^{-1} to 170 °C, held there for 55 min, and then temperature-programmed at 10 °C min^{-1} to 230 °C and held there for 33 min. Helium was the carrier gas with a column inlet pressure set at 30 psig and at a split ratio of 1:20. The injector temperature was set at 250 °C and detector at 270 °C. Injection volume was 0.5 μL. For GC-FID analysis, anhydrous milk fat with a certified fatty acid composition (reference material BCR-164, EU Commission, Brussels, Belgium, purchased from Fedelco Inc., Madrid, Spain) was used to determine the FAME response factors. For quantitative purposes, glyceryl tridecanoate (Sigma Chemical Co., St. Louis, MO, USA) was also used as internal standard.

Individual trans isomers were performed on the same system and column but with the following chromatographic conditions: the initial temperature of 100 °C was maintained for 3 min, then raised to 160 °C at a rate of 7 °C min and hold for 62 min, then raised to 220 °C at a rate of 2 °C and hold for 20 min. The split ratio was 1:50 and hydrogen was the carrier gas with a head pressure of 15 psig. The injector and detector temperatures were 250 °C. Tentative identification of trans-C18:2 and trans-C18:3 isomers was done by comparing the equivalent chain-length values of FAME obtained with those of reference oils: partially isomerised linseed oil FAME, refined rapeseed oil (BCR 686), partially hydrogenated sunflower seed oil (BCR-688) and a blend of palm oil and partially hydrogenated sunflower seed oil (BCR-687). Besides, FAME pure isomers (C18:1: cis-9; cis-13; trans-9; trans-11; trans-13) and PUFA mixtures (C18:2 and C18:3 mixtures) supplied by Supelco (Bellefonte, PN, USA) were also used as standards.

Silver-ion HPLC (Ag^+-HPLC)

Ag^+-HPLC separation of CLA methyl esters was carried out using an HPLC system (Shimadzu Vp Series, Duisburg, F.R. Germany) equipped with UV detector operated at 233 nm. FAMEs were separated using a ChromSpher 5 Lipid analytical column (4.6 mm i.d. × 250 mm stainless steel; 5 μm particle size; Varian-Chrompack International). The mobile phase, daily prepared, was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 mL min^{-1}. The flow was initiated 0.5 h prior to the sample injection, and the injection volume was 10 μL. Pure and mixed CLA FAME isomers from Nu-Chek Prep. Inc. (Elysian, MN, USA) were used as standards.

Quantitation of oxidation compounds

Quantitative analysis of total non-volatile oxidation compounds was carried out by separation of polar compounds by solid-phase extraction (SPE) and subsequent analysis by high-performance size-exclusion chromatography (HPSEC) (Márquez-Ruiz *et al.*, 1996). A volume of 2 mL of a hexane solution containing 50 mg of extracted oil and 1 mg of monostearin (Sigma), used as internal standard, was separated into two fractions by SPE. The first fraction, comprising the unoxidised triacylglycerols, was eluted with 15 mL of hexane/diethyl ether (90:10, v/v). The second fraction was eluted with 25 mL of diethyl ether and comprises the total non-volatile oxidation compounds, the internal standard, hydrolytic alteration compounds, i.e. diacylglycerols (DG) and free fatty acids (FFA), and polar unsaponifiable matter. Thus, the oxidation compounds are separated as compounds with higher polarity than that of the non-oxidised triglycerol molecules. After evaporation of the solvent in a rotary evaporator, the polar fraction was dissolved with 1 mL of diethyl ether. Efficiency of the separation was checked by TLC using hexane/diethyl ether/acetic acid (80:20:1, v/v/v) for the development of plates and exposure to iodine vapour to reveal the spots. The fraction of polar compounds was analysed in an HPSEC chromatograph equipped with a

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Rheodyne injector with 10-μL sample loop, a Waters 510 pump (Waters, Milford, MA, USA) and a Waters refractive index detector. The separation was performed on two 100 and 500 Å Ultrastyragel columns (25 cm × 0.77 cm i.d.) packed with porous, highly cross-linked styrene–divinylbenzene copolymers (film thickness 10 μm) (Agilent Technologies, Palo Alto, CA, USA) connected in series, with tetrahydrofuran (1 mL min^{-1}) as the mobile phase. The peaks resolved by HPSEC correspond to triacylglycerol dimers (TGD), oxidised triacylglycerol monomers (oxTGM), DG, monostearin and finally peaks corresponding to FFA of varying chain length and the polar unsaponifiable matter.

Determination of tocopherols

Tocopherols were determined by normal-phase HPLC with fluorescence detection according to IUPAC Standard Method 2.411 (IUPAC, 1992).

Detection of rancidity

Rancidity was determined by six untrained judges. Samples (20 g) corresponding to each temperature and period of storage were presented in Petri dishes simultaneously in randomised order, at a temperature of 23 ± 2 °C, and tested twice. The sample was considered rancid when clearly recognised rancid odour in intact samples was detected by the six judges (Márquez-Ruiz *et al.*, 2003a; Velasco *et al.*, 2006).

Statistical analysis

For statistical analysis, we used one-way analysis of variance (ANOVA) as well as multiple comparisons, performing the Bonferroni procedure for each fatty acid, tocopherol, group of DG and oxidation compounds, storage temperature and time period. SPSS package for Windows version 11 (SPSS, Chicago, IL, USA) was used. The level of statistical significance was set at 5% for all analyses.

Results and discussion

Fatty acid profile

Mean values and standard errors of the means for fatty acid composition in samples before and after 3-month storage are shown in Table 2. As no significant changes

Table 2 Fatty acid composition (weight per cent on fatty acid methyl esters) of infant formula initially and after 3-month storage at 25, 30 and 37 °C[a]

	Initial		25 °C		30 °C		37 °C	
Fatty acid	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
C4:0	0.06	0.001	0.06	0.001	0.06	0.003	0.06	0.001
C6:0	0.21	0.004	0.21	0.001	0.21	0.002	0.21	0.001
C8:0	1.72	0.021	1.74	0.006	1.74	0.006	1.76	0.012
C10:0	1.32	0.012	1.34	0.001	1.34	0.005	1.35	0.016
C12:0	9.62	0.087	9.74	0.020	9.71	0.037	9.78	0.142
C14:0	4.01	0.020	4.03	0.021	4.07	0.014	4.10	0.067
C15:0	0.04	0.001	0.04	0.001	0.04	0.004	0.04	0.001
C16:0	16.77	0.268	15.70	0.322	16.60	0.196	16.72	0.490
C16:1	0.11	0.001	0.11	0.001	0.11	0.007	0.11	0.002
C17:0	0.05	0.002	0.04	0.003	0.04	0.015	0.05	0.005
C18:0	3.31	0.030	3.25	0.015	3.30	0.017	3.30	0.033
transC18:1 (t4 to t12)	0.22	0.006	0.21	0.010	0.23	0.007	0.24	0.001
C18:1cis9	42.36	0.175	43.16	0.223	42.58	0.207	42.42	0.817
C18:2 cis9 cis12	17.24	0.082	17.42	0.130	17.12	0.053	16.98	0.109
C20:0	0.16	0.001	0.16	0.001	0.16	0.002	0.16	0.005
C20:1 cis9	0.17	0.004	0.17	0.002	0.17	0.001	0.17	0.002
C20:1 cis11	0.22	0.013	0.23	0.003	0.22	0.001	0.22	0.014
C18:3 n-3	1.37	0.020	1.38	0.017	1.34	0.007	1.32	0.036
C18:2 conjugated	0.02	0.001	0.02	0.002	0.02	0.003	0.02	0.001
C20:4 n-6	0.26	0.001	0.26	0.002	0.26	0.004	0.26	0.003
C20:5 n-3	0.11	0.002	0.11	0.001	0.11	0.001	0.10	0.001
Σ SFA	37.26	0.179	36.31	0.374	37.29	0.226	37.54	0.712
Σ MUFA	43.09	0.152	43.89	0.217	43.32	0.213	43.17	0.803
Σ PUFA	19.00	0.062	19.20	0.151	18.84	0.060	18.68	0.098

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[a]Data are expressed as means and standard error of the mean (SEM), $n = 3$.

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were found in any of the fatty acids along storage, results corresponding to 1 and 2 months have been omitted. As can be observed, the major fatty acid present in the infant formula was oleic acid (C18:1cis9) with about 42–43% of total FAME. The level of saturated fatty acids in the samples was about 37%, and approximately 70% of this level was palmitic acid (C16:0) and lauric acid (C12:0). Only a slight, not significant, decrease in PUFA of samples stored at 37 °C during 3 months could be noted.

As fatty acid composition did not change along storage under the conditions used, recommendations for levels and ratios of fatty acids were always fulfilled (Koletzko *et al*., 2005). Thus, the content of linoleic acid (C18:2 cis9-cis12) was about 860 mg per 100 kcal on average, well within the recommended range (300–1200 mg per 100 kcal). The content of α-linolenic acid (C18:3 n-3) was 66 mg $kcal^{-1}$ on average (a minimum of 50 mg per 100 kcal is recommended). As a result, the linoleic acid/α-linolenic acid ratio was about 13, which falls within the ratio established for infant formulae (between five and fifteen). The sum of myristic acid (C14:0) and lauric acid (C12:0) should not exceed 20% of total fat content in infant formulae and only 13–14% was found in the samples analysed in this study.

As expected, the total CLA content was low (0.02% of total FA), because mainly of the absence of milk fat and the presence only of vegetable oils in this infant formula, as reported in our previous work (Rodríguez-Alcala *et al*., 2007). Nevertheless, because of the present interest on the occurrence of CLA isomers in foods and their possible modifications or selective increase during industrial processing or storage (Rodríguez-Alcala & Fontecha, 2007), the distribution of these isomers was further analysed by Ag+-HPLC. The CLA isomer distribution (in relative proportions of total CLA) accounted for about 33% of C18:2 trans, trans isomers (the major isomers were C18:2 trans10, trans12 and C18:2 trans9, trans11), about 22% of C18:2 cis, cis isomers and about 45% of C18:2 cis, trans plus trans, cis isomers. The most biologically important isomers described were C18:2 cis9, trans11 (rumenic acid) and C18:2 trans10, cis12, which accounted for 24% and 8%, respectively. This isomer distribution was in the same range than that previously reported in infant formulae stored at 25 °C up to 3 years (Rodríguez-Alcala *et al*., 2007). Neither the CLA content nor the CLA isomer distribution showed significant differences during the storage period of samples at 25, 30 or 37 °C.

Mean values and standard errors of the means for trans isomers in samples before and after 3-month storage are shown in Table 3. As with fatty acid profile in Table 2 and for the sake of simplicity, only results for initial samples and 3-month-stored samples have been included in Table 3, given that no significant changes were found. Among trans fatty acids, trans-octadecenoic (t-C18:1) was the main isomer present, whereas trans-octadecadienoic (t-C18:2) and trans-octadecatrienoic acids (t-C18:3) were not detected. Overall, the total trans-C18:1 content was always lower than 0.2% of total fatty acids and did not increase during storage at 25, 30 or 37 °C.

Oxidation compounds and tocopherols

Quantitation of non-volatile oxidation compounds constitutes a direct and global measurement of major oxidised compounds and has proven to be suitable and very useful when applied to powdered ingredients and infant formulae (Márquez-Ruiz *et al*., 2003a; Velasco

Table 3 Trans monoene isomer composition (milligrams per 100 g of extracted lipids) in infant formula initially and after 3-month storage at 25, 30 and 37 °C[a]

	Initial		25 °C		30 °C		37 °C	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
C16:1 trans	5.83	0.258	6.40	0.364	5.61	0.364	5.89	0.404
C18:1 trans								
trans 4–5	19.83	1.472	15.12	2.809	15.91	2.277	14.52	2.558
trans 6–8	40.46	2.497	36.96	1.779	38.90	1.932	37.91	0.726
trans 9	43.79	1.912	43.26	2.085	45.26	4.770	46.86	2.890
trans 10	46.50	0.717	52.32	1.800	55.23	2.195	52.31	1.140
trans 11	16.77	1.226	16.95	2.766	18.04	1.403	19.81	1.792
trans 12	28.15	0.714	30.70	1.999	26.13	2.116	31.13	1.295
trans 13–14	11.10	0.920	10.57	0.299	11.70	0.295	10.63	0.419
trans 15	12.07	1.218	10.10	0.615	11.13	0.234	10.08	0.314
trans 16	0.22	0.008	0.22	0.003	0.23	0.008	0.23	0.001
C18:1 total trans (%)	0.20	0.015	0.15	0.028	0.16	0.023	0.14	0.026

[a]Data are expressed as means and standard error of the mean (SEM), $n = 3$.

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Table 4 Oxidised triacylglycerol monomers (oxTGM), triacylglycerol dimers (TGD), diacylglycerols (DG) and free fatty acids (FFA) (weight per cent on extracted oil) in infant formula initially and after 3-month storage at 25, 30 and 37 °C[a]

	Initial		25 °C		30 °C		37 °C	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
oxTGM	1.1	0.01	1.3	0.09	1.3	0.10	1.4	0.18
TGD	0.5	0.06	0.5	0.05	0.5	0.07	0.6	0.05
DG	2.7	0.05	2.8	0.17	2.8	0.11	2.9	0.04
FFA	0.6	0.03	0.7	0.04	0.6	0.04	0.6	0.03

[a]Data are expressed as means and standard error of the mean (SEM), $n = 3$.

Table 5 Tocopherols (Toc) (milligrams per kilogram of extracted lipids) in infant formula initially and after 3-month storage at 25, 30 and 37 °C[a]

	Initial		25 °C		30 °C		37 °C	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Total Toc	372	6.5	365	3.6	351	9.1	374	6.3
α-Toc	177	6.2	176	3.1	167	4.7	174	12.0
β-Toc	14	0.7	15	2.8	13	2.7	13	0.3
γ-Toc	135	3.1	139	3.1	130	4.8	139	5.2
δ-Toc	45	1.2	44	5.4	42	1.2	43	1.6

[a]Data are expressed as means and standard error of the mean (SEM), $n = 3$.

et al., 2008). Table 4 shows data of oxTGM, TGD, DG and FFA in initial samples and samples stored at 25, 30 or 37 °C for 3 months. The group of oxTGM includes the primary oxidation compounds formed (hydroperoxides) and secondary oxidation compounds (alcohols, ketones, epoxides, etc.) in monomeric TG structures, while TGD formation marks the start of the advanced oxidation stage (Márquez-Ruiz *et al.*, 2003a,b; Márquez-Ruiz & Dobarganes, 2005). No significant differences were found in oxidation compounds (oxTGM and TGD) or in hydrolysis products (DG and FFA), along the storage period. The values reported are within those normally found in refined vegetable oils.

Table 5 shows data of total tocopherols and specific values for α, β, γ and δ isomers. Infant formulae contain tocopherols derived from the vegetable oils used and from their specific addition during their manufacture. In this study, α-tocopherol and γ-tocopherol constituted the major fraction of the total tocopherol content. European legislation requires a minimum content of 0.5 α-tocopherol equivalents per 100 kcal in infant formulae (Koletzko *et al.*, 2005). No significant differences were found after storage, and the total vitamin E activity exceeded by far the minimum content established by regulations European law in all samples. As commented in the Introduction, results reported on changes of tocopherols after storage of infant formulae at ambient temperatures are variable (Albala-Hurtado *et al.*, 2000; Rodríguez-Alcala *et al.*, 2007; Chávez-Servín *et al.*, 2008b), but this is expected from the different conditions of oxygen availability and infant formulae compositions (fatty acid composition, presence of antioxidants and/or prooxidants) used.

Even though no significant differences were found in oxidation compounds or tocopherol levels, rancidity was clearly perceived by the six judges in samples stored at 37 °C during 3 months. To gain insight into the oxidation status of samples, they were further analysed following separate extraction of the free oil fraction and the encapsulated oil fraction. The free or non-encapsulated oil fraction is a minor lipid fraction that can be easily extracted by simply washing with hexane (see Methods). Even though it constituted only 7.5% of total oil in these samples, its analysis can be of great relevance because the free oil fraction may be more susceptible to oxidation than the oil fraction embedded in the matrix (encapsulated oil). If oxidised, rancidity of the more accessible free oil fraction can be easily perceived (Márquez-Ruiz *et al.*, 2003a; Velasco *et al.*, 2006).

Figure 1 shows the results obtained for total non-volatile oxidation compounds, i.e., the sum of oxTGM and dimers, in the free oil fractions along storage. No significant changes were observed in the amounts of free oil extracted along storage, with values of 7.5% on average and coefficients of variation lower than 4%. As can be observed, there was a significant increase in oxidation compounds along storage already noted after 1 month at 37 °C. After 3 months, values were practically double than those initially found for all temperatures, and higher than 4% in the case of 37 °C. These results agreed well with the detection of rancidity in these samples and show the necessity to extract the

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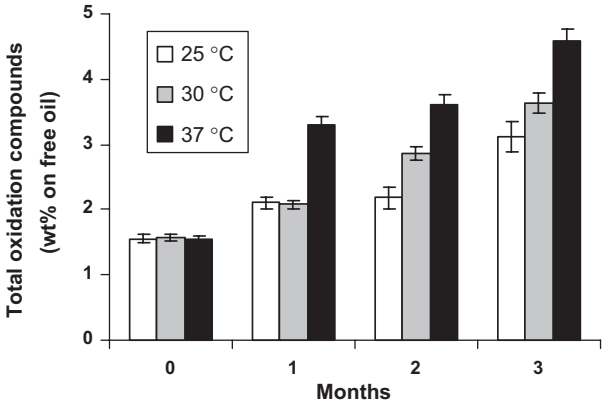

Figure 1 Evolution of total oxidation compounds in free oil fractions extracted from infant formula stored at 25, 30 and 37 °C.

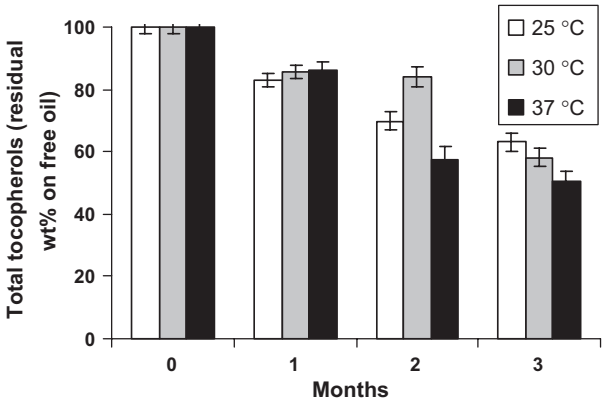

Figure 2 Evolution of total tocopherols in free oil fractions extracted from infant formula stored at 25, 30 and 37 °C.

minor free oil fraction in infant formulae to evaluate lipid oxidation.

Values of total tocopherols in free oil fraction along storage were consistent with the data obtained for total oxidation compounds. Thus, a significant decrease occurred from the first month at all temperatures, and losses between 40% and 50% of total tocopherols were found after 3 months (Fig. 2). Although the free oil fraction was still well protected by remaining antioxidants in all samples, this finding clearly suggests that free oil was more susceptible to oxidation than encapsulated oil. However, oxidation of the free oil fractions was masked when the total oil was analysed because of the low contribution of surface oil to the total oil.

The most important conclusions of the present study are, on the one hand, that infant formula stored at 25, 30 or 37 °C for 3 months did not show significant changes in fatty acid composition profile, including CLA isomers, and neither trans isomers nor non-volatile oxidation compounds were formed in significant amounts. On the other hand, analysis of free oil fractions revealed that rancidity, when detected, was because of their preferential oxidation. Overall, even though oxidation levels found in free oil fractions may be very low in terms of nutritional and safety significance, their detection should not be disregarded because oxidation in the free oil may lead to rancidity and determine the shelf-life of infant formulae.

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3.6.- Compuestos de oxidación volátiles en un aceite rico en ácido linoleico conjugado.

Volatile oxidation compounds in a conjugated linoleic acid-rich oil.

García-Martínez María del Carmen, Márquez-Ruiz Gloria, Javier Fontecha y Michael H. Gordon.

Food Chemistry, 2009; 113: 926-931.

Resumen:

En este estudio se analizaron los compuestos de oxidación volátiles formados en un aceite funcional rico en ácido linoleico conjugado (CLA) comercial, y se compararon los resultados con los obtenidos en aceite de cártamo (rico en ácido linoleico, LA). Se utilizaron muestras de aceite y triacilgliceroles puros, obtenidos tras la eliminación de tocoferoles y compuestos menores, se oxidaron a 60 ºC y se analizaron los compuestos de oxidación volátiles mediante microextracción en fase sólida-cromatografía de gases con detector de ionización de llama y espectrometría de masas. Los resultados mostraron que, como era esperado, el hexanal fue el mayor compuesto de oxidación volátil encontrado en el aceite y triacilgliceroles ricos en LA, mientras que el hexanal y el heptanal fueron los compuestos más abundantes en el aceite y triacilgliceroles ricos en CLA. Además, las muestras ricas en CLA también mostraron altas cantidades de trans-2-octenal y trans-2-nonenal, y este último junto con el heptanal no se encontraron en las muestras ricas en LA. Los resultados para las muestras de CLA son difíciles de interpretar, ya que los principales compuestos volátiles encontrados no son los procedentes de los hidroperoxidos teóricamente formados en la oxidación del CLA. Podrían proceder en parte de los dioxetanos provenientes de 1,2-cicloadiciones del CLA con oxígeno. En general, los resultados obtenidos apoyan la evidencia de que los mecanismos de oxidación del CLA difieren de los del LA. También se concluye que la determinación de heptanal puede ser un marcador útil del progreso de oxidación en aceites ricos en CLA.

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Volatile oxidation compounds in a conjugated linoleic acid-rich oil

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ABSTRACT

In this study, volatile oxidation compounds formed in a commercial conjugated linoleic acid (CLA)-rich oil were quantified and results compared to those found in safflower oil (rich in linoleic acid, LA). Intact oil samples and pure triacylglycerols obtained following elimination of tocopherols and minor compounds were oxidised at 60 °C, and volatile oxidation compounds were analysed by solid phase microextraction-gas chromatography with flame ionisation detector and mass spectrometer. Results showed that while, as expected, hexanal was the major volatile oxidation compound found in oil and triacylglycerols rich in LA, both hexanal and heptanal equally were the most abundant compounds in oil and triacylglycerols rich in CLA. Besides, samples rich in CLA also showed significantly high quantities of *trans*-2-octenal and *trans*-2-nonenal and the latter, along with heptanal, were absent in samples rich in LA. Results for CLA samples were not easy to interpret since major volatiles found are not expected from theoretically stable hydroperoxides formed in CLA and could in part derive from dioxetanes coming from 1,2-cycloadditions of CLA with oxygen. Overall, results obtained support evidence that oxidation mechanisms of CLA may differ than those of LA. Also, it was concluded that heptanal determination could serve as a useful marker of oxidation progress in CLA-rich oils.

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1. Introduction

Conjugated linoleic acids (CLAs) are a group of positional and geometric isomers of linoleic acid (LA) that are widely distributed in various foods, primarily in dairy products and meat from ruminants (Fritsche et al., 1999). There has been considerable research into CLA in the last two decades, in areas ranging from cancer to obesity. Numerous researchers have reported that CLA exerts anticarcinogenic activity in different stages of tumour development (Ha, Grimm, & Pariza, 1987; Ha, Storkson, & Pariza, 1990). Anticarcinogenic properties, as well as antiatherogenic effects, have been attributed to *cis*-9, *trans*-11 linoleic acid, also known as rumenic acid (Masso-Welch et al., 2004), whereas *cis*-10, *trans*-12 linoleic acid seems responsible for the observed weight loss and muscle-mass enhancement effects (Malpuech-Brugere et al., 2004; Whigham, Watras, & Schoeller, 2007). In nature, the most abundant isomer is *cis*-9, *trans*-11 linoleic acid, whereas typical commercial CLA preparations intended for human use consist almost entirely (i.e., >80%) of the two biologically active isomers in approximately equal amounts (i.e., about 45% each). There is no evidence that consumption of such CLA preparations, at 3–6 g/day, could induce adverse effects in healthy humans (Gaullier et al., 2005).

However, safety concerns regarding the use of CLA in humans persist and need further investigation (Park & Pariza, 2007). In this context, it is essential to gain insight into the occurrence of oxidised compounds in commercial CLA preparations, which could lead to adverse physiological effects in cardiovascular and cancer processes (Dobarganes & Márquez-Ruiz, 2003), which are precisely the same targets for potential health benefits of CLA.

So far, studies on CLA oxidation are scarce and contradictory, and it is generally agreed that oxidation pathways of CLA are unclear and that the mechanisms involved remain controversial (Brimberg & Kamal-Eldin, 2003; Eulitz, Yurawecz, & Ku, 1999; Hämäläinen, Sundberg, Hase, & Hopia, 2002; Hämäläinen et al., 2001; Luna, De La Fuente, Salvador, & Márquez-Ruiz, 2007; Yurawecz, Delmonte, Vogel, & Kramer, 2003; Yurawecz et al.,1997). Some authors (Brimberg & Kamal-Eldin, 2003; Eulitz et al., 1999; Hämäläinen et al., 2001; Luna et al., 2007; Yurawecz et al., 2003, 1997) have suggested that, in contrast to the case of linoleate, hydroperoxides are only minor products in the oxidation of conjugated linoleate. Therefore, the methods normally used to control lipid oxidation in foods, e.g., peroxide value and thiobarbituric acid reactive substances, may not indicate the real level of oxidation Suzuki, Abe, & Miyashita, 2004) and provide misleading results when comparing oxidative stability of CLA and LA. This could be in great part due to formation of polymers from the very beginning of the oxidation process (Brimberg & Kamal-Eldin, 2003; Luna et al., 2007).

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In relation to volatile oxidation compounds formed in CLA, Yurawecz and coworkers reported the identity of those found in methyl CLA, following analyses by GC/MS and GC/MI/FITR that mainly showed the important contribution of methyl furan acids (Yurawecz et al., 2003, 1997). Quantitative evaluation of CLA volatile oxidation products has not been reported so far, even though such data would help unravel the possible differences in oxidation mechanisms between CLA and its non-conjugated counterpart LA.

The aim of this work was to study formation of volatile oxidation compounds in a commercial CLA preparation (Tonalin® TG 80 oil, rich in CLA), as compared to that in safflower oil (rich in LA). Intact oil samples and purified triacylglycerols obtained following elimination of tocopherols and minor compounds were oxidised at 60 °C for different periods of time and volatile oxidation compounds were analysed by solid phase microextraction-gas chromatography (SPME-GC).

2. Materials and methods

2.1. Materials

Tonalin® TG 80 oil (TO) was a gift from Santiveri SA (Barcelona, Spain), and was originally acquired from Cognis Nutrition and Health (Cincinnati, OH). It contained 0.1% mixed tocopherols (E306) added after isomerisation. Refined safflower oil (SO) was purchased from Interfat SA (Barcelona, Spain). TO and SO were passed through an aluminium oxide (activated at 200 °C for 3 h) column twice in order to remove tocopherols and minor polar compounds as described by Yoshida, Kondo, and Kajimoto (1992). Thereby, purified triacylglycerols were obtained (TO-TAG and SO-TAG). Solid phase microextraction (SPME) fibres coated with 75 μm Carboxen™/polydimethylsiloxane (CAR/PDMS) were obtained from Supelco (Bellefonte, PA). Tridecanoin was purchased from Sigma Chemical Co. (St. Louis, MO) and bromobenzene from Fluka Chemika (Buchs, Switzerland). Other chemicals and reagents used were of analytical grade and obtained from local suppliers.

2.2. Oxidation procedure

Triplicate samples of TO and SO (14 g each) were stored for 10 days in 20 ml dark vials at 60 °C and aliquots (1 g) were withdrawn at 24 h intervals, for SPME-GC and tocopherol analyses. In a second experiment, triplicate samples of TO-TAG and SO-TAG (3.5 g each) were stored for 21.5 h in the same type of vials and aliquots (0.8 g) were withdrawn during the oxidation period for SPME-GC analyses.

2.3. Analytical methods

2.3.1. Determination of tocopherols

Tocopherols were determined by normal-phase HPLC with fluorescence detection, according to IUPAC Standard Method 2.411 (IUPAC, 1992).

2.3.2. Analysis of fatty acid composition

Fatty acid composition was determined by GC-FID analysis. The oils containing added tridecanoin as internal standard were converted into fatty acid methyl esters (FAMEs) using 2 M KOH in methanol, as described by International Standard ISO-IDF (ISO, 2002). FAMEs were analysed on a Perkin–Elmer chromatograph (Autosystem Model, Beaconsfield, UK) with an FID detector. Fatty acids were separated using a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm ID × 0.2 μm film thickness, Varian Chrompack, Middelburg, The Netherlands). The column was held at 100 °C for 1 min after injection, temperature-programmed at 7 °C/min to 170 °C, held there for 55 min, and then temperature-programmed at 10 °C/min to 230 °C and held there for 33 min. Helium was the carrier gas with a column inlet pressure set at 30 psig (214 kPa) and a split ratio of 1:20. The injector temperature was set at 250 °C and detector temperature was set at 270 °C. The injection volume was 0.5 μl. CLA isomers were identified and quantified as in previous studies (Rodríguez-Alcalá & Fontecha, 2007).

2.3.3. Analysis of volatiles

SPME was performed using a 75 μm carboxen/polydimethylsiloxane (CAR/PDMS) fibre mounted in a SPME manual holder assembly (Supelco). The fibre was conditioned for 30 min in the injection port of the GC at 250 °C as recommended by the manufacturer. A 1 g aliquot of sample was weighed in a 20 ml dark vial. Ten microlitres of internal standard solution (1.495 mg/ml of bromobenzene in methanol) and a stirring bar were added. The vial was capped with a PTFE septum (Q_{mx} Laboratories, Thaxted, UK) and placed in a water bath (60 °C) on a magnetic stirrer, and the sample was equilibrated for 5 min at the required temperature before SPME sampling. The septum was manually pierced with the SPME needle and the fibre was exposed to the sample headspace for 30 min.

Volatiles were analysed with a Hewlett–Packard (Palo Alto, CA) 5890 series II gas chromatograph equipped with FID detector, split/splitless injector and a CP-Sil 8 CB low bleed/MS fused-silica capillary column (5% phenyl/95% PDMS; 60 m × 0.25 mm ID, 0.25 μm film thickness; Varian Chrompack). A section of column nearest to the injection port was cooled in a beaker of powdered solid carbon dioxide to cryofocus the volatiles. Volatile compounds were desorbed from the SPME fibre onto the front of the column for 3 min. The injection port was in splitless mode, and split flow was programmed to turn on after 0.5 min. The temperature of the injector was 250 °C. After fibre desorption, the solid carbon dioxide was removed and the GC programme started. The oven was maintained at 40 °C for a further 2 min and then the temperature was raised to 120 °C at 4 °C/min and then at 20 °C/min to 250 °C, and held at 250 °C for 10 min. The FID temperature was 280 °C. Helium at 16 psig was used as the carrier gas. *n*-Alkanes (C_5–C_{25}) were run under the same conditions to obtain linear retention index (LRI) values for the components (Hashizume, Gordon, & Mottram, 2007).

2.3.4. Identification of volatile oxidation compounds

The identification of compounds was performed on a Hewlett–Packard (Palo Alto, CA, USA) 5972 mass spectrometer, coupled to a 5890 Series II gas chromatograph and a G1034C Chemstation.

The mass spectrometer was operated in electron impact mode with an electron energy of 70 eV and an emission current of

Table 1
Fatty acid composition (weight percent of fatty acid methyl esters) and tocopherol composition (mg kg^{-1}) of safflower oil (SO) and Tonalin® oil (TO)

	SO	TO
Fatty acid composition (%)		
16:0	7.2 ± 0.24	2.4 ± 0.12
18:0	2.6 ± 0.09	2.6 ± 0.11
18:1	13.7 ± 0.52	14.2 ± 0.57
18:2 *c*9, *c*12	74.7 ± 0.68	0.5 ± 0.06
18:2 *c*9, *t*11 (CLA)		38.2 ± 0.72
18:2 *t*10, *c*12 (CLA)		38.6 ± 0.73
Others	1.8 ± 0.11	3.5 ± 0.22
Tocopherols (mg kg^{-1})		
α	266 ± 12.58	28 ± 1.95
β		324 ± 17.13
γ		215 ± 13.08

Data are expressed as means ± standard deviations ($n = 3$). *c* = *cis*; *t* = *trans*; and CLA = conjugated linoleic acid.

50 μA. The ion source was maintained at 170 °C. The mass spectrometer scanned from *m*/*z* 29 to *m*/*z* 400 at 1.9 scan/s. Compounds were identified by first comparing their mass spectra with those contained in the National Institute of Standards and Technology

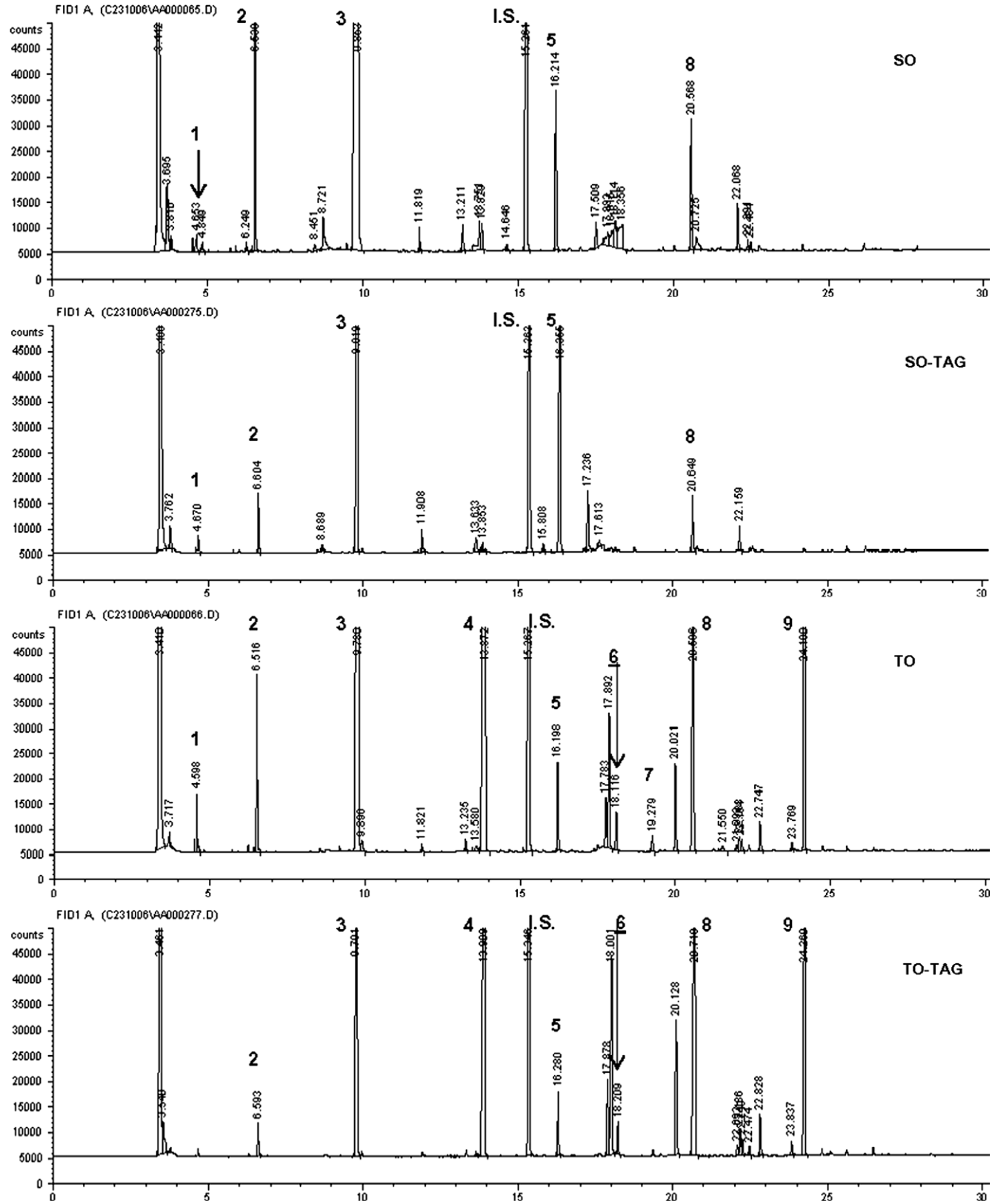

Fig. 1. GC-chromatograms of: SO, oxidised safflower oil at 10 days; SO-TAG, oxidised safflower oil triacylglycerols at 21.5 h; TO, oxidised Tonalin® oil at 10 days and TO-TAG, oxidised Tonalin® oil triacylglycerols at 21.5 h. IS: internal standard. Volatile oxidation compounds: 1, hexane; 2, pentanal; 3, hexanal; 4, heptanal; 5, *trans*-2-heptenal; 6, octanal; 7, limonene; 8, *trans*-2-octenal and 9, *trans*-2-nonenal.

(NIST) followed by comparing LRI values with *n*-alkanes (C_5–C_{25}) (Hashizume et al., 2007).

2.3.5. Statistical analysis

Data reported were obtained from triplicate samples and expressed as mean and relative standard deviation or standard error of the mean. Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA) was used for data analyses.

3. Results and discussion

Fatty acid analysis (Table 1) showed that 76.8% of TO composition was CLA, containing approximately equal amounts of the *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA isomers. SO, which is the natural oil source for preparation of TO, contained a very similar concentration of LA (74.7%) to that of total CLA in TO, and showed no detectable amounts of CLA. This fact, along with the similar concentrations of oleic acid (about 14%) in both oils, made them quite suitable for comparison of the formation of volatile oxidation compounds in CLA and LA. Table 1 also includes the tocopherol composition of the oils. TO contained more than twice the concentration of total tocopherols (567 mg/kg) than SO (266 mg/kg). Only the α-tocopherol isomer was present in SO, while γ- and δ-tocopherol isomers were the most abundant in TO. In order to establish differences in oxidation products due to differences in fatty acid composition rather than antioxidant content, samples tested included those stripped of tocopherols. In this latter case, samples contained triacylglycerols almost exclusively since alumina treatment eliminated other minor polar compounds such as diacylglycerols, monoacylglycerols and free fatty acids. In the stripped oils, total absence of tocopherols and no significant changes in FAME composition were confirmed in TO-TAG and SO-TAG (data not shown).

Fig. 1 shows GC chromatograms of volatile oxidation compounds from oxidised TO and SO, TO-TAG and SO-TAG. As can be observed, a similar profile of volatile decomposition products was found for the commercial oils TO and SO as compared to their corresponding purified triacylglycerol fractions TO-TAG and SO-TAG, in spite of the differences in minor compound composition (including tocopherols) and oxidation time. In SO and SO-TAG, the volatile profile was close to that expected from the cleavage of the alkoxyl radicals formed from the hydroperoxides of autoxidised linoleic acid and linoleic acid-rich oils, which has been extensively reported (Frankel, 1986, 2005). Thus, hexanal was the main volatile oxidation product, which is derived from the 13-hydroperoxide, which is one of the major hydroperoxides formed by autoxidation of linoleic acid. Pentanal, *trans*-2-heptenal and *trans*-2-octenal are other major volatiles formed during oxidation of SO and SO-TAG, also produced by decomposition of the most abundant linoleic acid hydroperoxides (Frankel, 1986, 2005). However, the volatile profile of oxidised TO and TO-TAG was characterised by the joint occurrence of two major volatile oxidation products, i.e., hexanal and heptanal, while the latter compound was absent in oxidised SO and SO-TAG. Another important difference was the presence of *trans*-2-nonenal, as an additional relevant peak, in TO and TO-TAG, while it was totally absent in SO and SO-TAG. According to the hydroperoxide theory, formation of hexanal and pentanal is predictable from the expected major 13-hydroperoxides formed in both *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, both isomers present in equal amounts in TO and TO-TAG. In contrast, the presence of heptanal and *trans*-2-nonenal, as well as that of other minor peaks, is not easily accounted for. Heptanal could come from β-scission of the alkoxyl radical formed from 12-hydroperoxy-*trans*-8, *trans*-10-octadecadienoate, in turn reported to be one of the hydroperoxides formed in oxidised *cis*-9, *trans*-11 CLA (Hämäläinen et al., 2002). However, *trans*-2-nonenal would be a

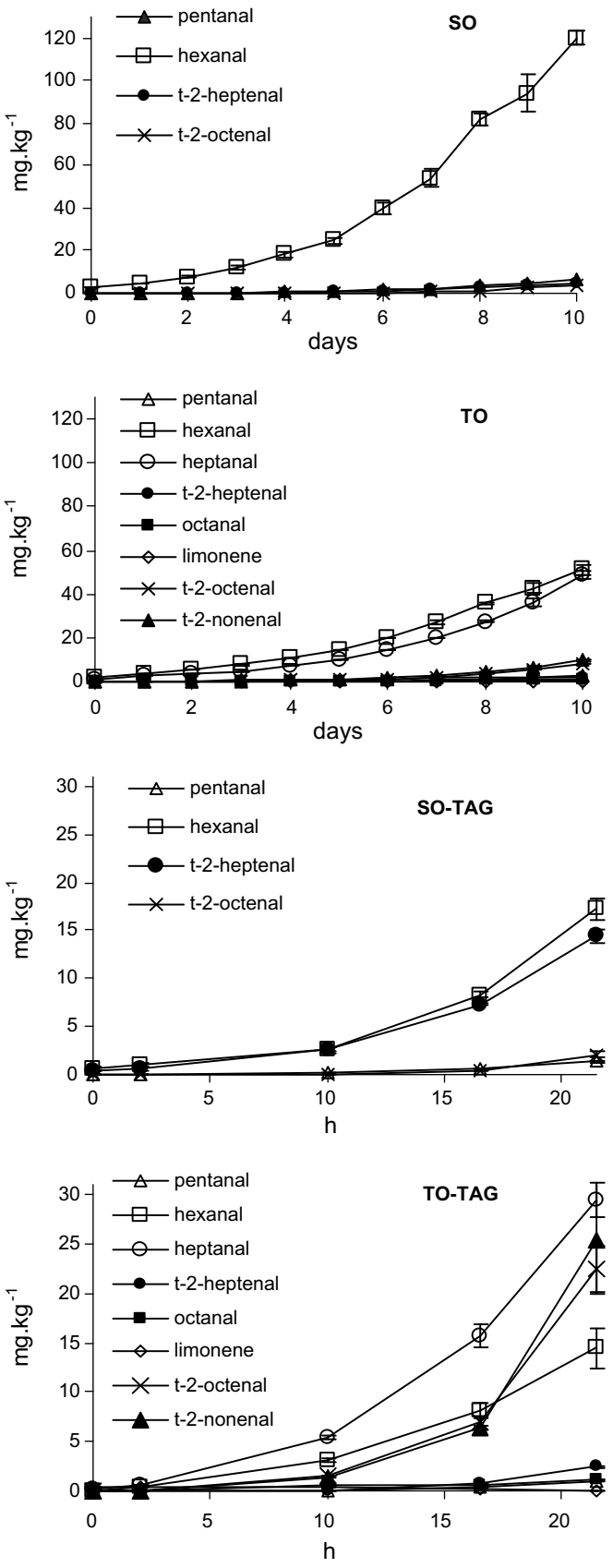

Fig. 2. Time course of formation of main volatile oxidation compounds. Error bars indicate standard errors of the means ($n = 3$). SO, safflower oil; TO, Tonalin® oil; SO-TAG, safflower oil triacylglycerols; and TO-TAG, Tonalin® oil triacylglycerols.

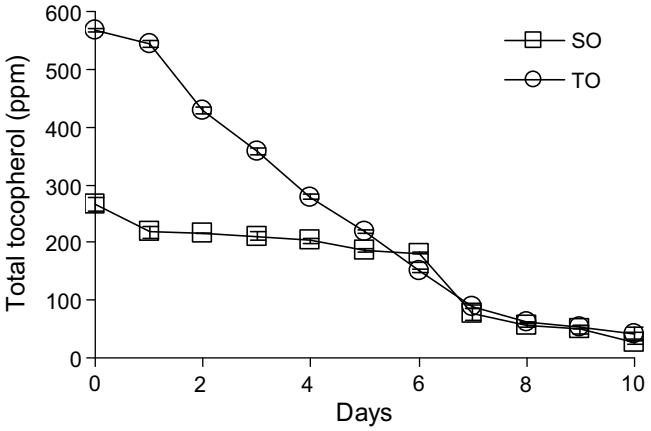

Fig. 3. Evolution of total tocopherols during the oxidation of oils. Error bars indicate standard errors of the means ($n = 3$). SO, safflower oil; and TO, Tonalin® oil.

product derived from the 10-hydroperoxy-*trans*-8, *trans*-11-octadecadienoate or 10-hydroperoxy-*trans*-8, *cis*-11-octadecadienoate in oxidised *cis*-9, *trans*-11 CLA, and it has been reported such hydroperoxides are unlikely to be formed because of the instability of the resonance structures in which double bonds are not conjugated in the pentadienyl radicals (Hämäläinen et al., 2002). Likewise, *trans*-2-octenal would come from the supposedly unexpected 11-hydroperoxy-9,12-octadecadienoate (Tallman, Pratt, & Porter, 2001; Tallman, Roschek, & Porter, 2004) from both *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, which would also contribute to formation of further amounts of heptanal. An alternative route of formation for the oxidation volatile compounds found in CLA could be based on the mechanisms proposed by Yurawecz and coworkers. They suggested that CLA may undergo 1,2-cycloadditions with oxygen, resulting in dioxetanes that would lead to volatile formation (Yurawecz et al., 2003). Thus, they found heptanal and 2-nonenal from the autoxidation of methyl *cis*-9, *trans*-11 linoleate and proposed that heptanal would be a scission product of a 11,12-dioxetane, whereas 2-nonenal would result from the scissions of 9,10-dioxetane.

The time course of formation of the main oxidation volatiles for SO, SO-TAG, TO and TO-TAG is shown in Fig. 2. It is clear that the volatile concentrations were very reproducible, as shown by the small error bars. Comparison of SO with TO showed that, from the beginning, the main volatiles were hexanal for SO and both hexanal and heptanal for TO. In samples stripped of tocopherols and minor polar compounds, i.e., SO-TAG and TO-TAG, the nature of the volatiles detected did not change. However, interestingly, some significant differences were observed in the relative amounts present in the purified triacylglycerol sample. In SO-TAG, in contrast to SO, *trans*-2-heptenal was a major volatile compound from the beginning and was found in similar concentrations to hexanal. In TO-TAG, in contrast to TO, formation of heptanal was faster and at higher levels than that of hexanal. Also, heptanal increases were closely followed by those of *trans*-2-nonenal and *trans*-2-octenal, which even exceeded the amount of hexanal at times greater than 17 h. Overall, results showed that, as for hexanal in the case of LA-rich oils, heptanal can be used as a marker of the oxidation progress in CLA-rich oils.

The main difference affecting volatile formation from SO and TO as compared with their counterparts SO-TAG and TO-TAG, respectively, is that both SO and TO contained tocopherols during the oxidation period tested, as can be observed in Fig. 3. It has been reported that, in the presence of tocopherols, relative abundance of the hydroperoxides formed in methyl linoleate and methyl linolenate at 40 °C may change as compared to oxidation in the absence of tocopherols (Peers, Coxon, & Chan, 1981, 1984). Specifically regarding volatile formation, α-tocopherol was found to affect the amount and distribution of the main volatiles formed in thermally-oxidised methyl linoleate (Frankel & Gardner, 1989). The contribution of reactions other than hydroperoxide formation in the case of TO and TO-TAG, as suggested by Yurawecz and coworkers, is likely and may affect the abundance and relative amounts of the volatiles formed. In this context, it has been recently demonstrated that pure methyl conjugated linoleate polymerised rapidly and formation of hydroperoxides was negligible at 30 °C (Luna et al., 2007).

Storage of the TO-TAG for 21.5 h was sufficient for the total volatile content of the sample to be comparable with that of the SO and TO samples stored for 10 days (Table 2). The concentration of total volatile oxidation compounds was somewhat higher in SO (153 ppm) than in TO (132 ppm) but it was much higher in TO-TAG (125 ppm) than in SO-TAG (41.7 ppm). Despite the differences in proportions of volatiles found between the original oils and purified triacylglycerols, especially between TO and TO-TAG, which was discussed above, it is clear that heptanal was undoubtedly the major volatile oxidation product in both samples.

Both SO and TO still contained residual amounts of tocopherols at 10 days (Fig. 3). In other words, data obtained for TO and SO during the oxidation period tested corresponded to the induction period. Also, it is important to note that tocopherol loss was much more rapid in TO than in SO since TO contained initially more than twice the concentration of tocopherols than that of SO and both were almost devoid of tocopherols at 10 days. This difference in the rate of loss of tocopherols reflects the greater susceptibility to oxidation of CLA compared to LA, as shown in the formation of

Table 2
Quantification of major volatile oxidation compounds (mg kg^{-1}) in samples at the end of the oxidation experiments

	SO (10 days)			TO (10 days)			SO-TAG (21.5 h)			TO-TAG (21.5 h)		
	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD
Hexane	0.4	0.01	2.9	0.8	0.12	15.7	0.5	0.04	9.0			
Pentanal	6.5	0.48	7.4	2.7	0.31	11.7	1.4	0.13	9.2	1.0	0.03	2.8
Hexanal	120	5.21	4.3	51.1	4.59	9.0	17.2	1.49	8.7	14.5	2.87	19.8
Heptanal				48.5	3.16	6.5				29.4	2.45	8.3
trans-2-Heptenal	4.7	0.36	7.6	2.1	0.05	2.6	14.4	1.01	6.9	2.5	0.20	8.1
Octanal	1.2	0.69	58.3	0.9	0.04	4.3				1.2	0.06	4.7
Limonene				0.4	0.01	0.8						
trans-2-Octenal	4.0	0.36	9.0	8.5	0.31	3.7	2.1	0.41	19.8	22.5	3.20	14.3
trans-2-Nonenal	0.2	0.08	0.1	10.1	0.53	5.3				25.4	7.77	30.5
Others	16.3	1.40	8.7	7.3	0.40	5.5	6.1	0.40	6.7	28.5	7.40	26.1
Total volatiles	153	8.66	5.6	132	9.63	5.7	41.7	3.63	8.7	125	11.54	9.2

Values are expressed as means, standard deviations (SD) and relative standard deviations (RSD, %). SO, safflower oil; TO, Tonalin® oil; SO-TAG, safflower oil triacylglycerols; and TO-TAG, Tonalin® oil triacylglycerols.

volatiles in samples without tocopherols (Table 2). Once the tocopherols were exhausted, the proportion of volatiles formed in TO and SO after 10 days storage increased to values closer to those found in TO-TAG and SO-TAG.

Overall, the results obtained in this study provide quantitative data on the formation of volatile oxidation products in CLA-rich oils, which is of great value for two reasons. The data allows an insight into the oxidation mechanisms of conjugated dienes and, in addition, it provides a useful analytical tool for monitoring the progress of oxidation in CLA-rich oils. More research is needed into aspects concerning CLA oxidation, since CLA-rich oils are nowadays used as a functional ingredient in a growing number of foods and dietary supplements. Studies are currently underway in our laboratory to determine the formation of volatile and non-volatile oxidation products in oxidised TO and SO at different temperatures.

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3.7.- Oxidación de un aceite funcional rico en CLA: determinación de compuestos volátiles y no volátiles.

Oxidation of a functional, CLA-rich oil: determination of volatile and non-volatile compounds.

Gloria Márquez-Ruiz, Francisca Holgado, María Victoria Ruiz-Méndez, Joaquín Velasco y María del Carmen García-Martínez.

European Food Research and Technology, 2016; 242: 1993-2000.

Resumen:

El objetivo de este trabajo ha sido monitorizar y comparar la formación de compuestos de oxidación volátiles y no volátiles en el aceite Tonalin® (TO) rico en ácido linoleico conjugado (CLA) y en aceite de cártamo (SO) rico en ácido linoleico (LA) a 40ºC en oscuridad. En el caso del TO, la formación de hidroperóxidos fue muy minoritaria y los primeros y principales compuestos formados fueron polímeros. Cuando los tocoferoles se agotaron, el SO mostró 152 meq O_2/kg de índice de peróxidos y 3% de polímeros, valores consistentes con el progreso de oxidación típico en aceites insaturados en estas condiciones, mientras que el TO sólo mostró 19 meq O_2/kg de índice de peróxidos y una elevada concentración de polímeros, 15%. Respecto a la composición de compuestos volátiles, el SO mostró los compuestos esperados, provenientes de la división de los radicales alcoxilo derivados de los hidroperóxidos del LA, siendo el hexanal el principal compuesto. Sin embargo, el perfil de volátiles del TO se caracterizó por la presencia de heptanal y t-2-nonenal, ausentes en el SO. Una ruta de formación alternativa para estos compuestos de oxidación volátiles encontrados en TO podría ser la escisión de los dioxietanos provenientes de 1,2 cicloadiciones del CLA con oxígeno. En esencia, los resultados obtenidos en este estudio apoyan que la cinética de oxidación de los aceites ricos en CLA difiere sustancialmente de la teoría de formación de hidroperóxidos como compuestos primarios de oxidación. La oxidación del CLA parece ocurrir preferentemente mediante la adición de radicales peroxilo a dobles enlaces durante las reacciones de propagación, favoreciendo de esta manera la formación de peróxidos oligoméricos desde los primeros estadíos de la degradación lipídica.

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ORIGINAL PAPER

Oxidation of a functional, CLA-rich oil: determination of volatile and non-volatile compounds

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Abstract The objective of this work was to monitor and compare formation of non-volatile and volatile oxidation compounds in a conjugated linoleic acid (CLA)-rich oil, Tonalin® oil (TO) and a linoleic acid (LA)-rich oil, safflower oil (SO) at 40 °C in the dark. In the TO, formation of hydroperoxides was negligible and the first and major compounds formed were polymerization products. When tocopherols were exhausted, the SO showed 152 meq O_2/kg oil and 3 % polymers, values which are consistent with the expected progress of oxidation in unsaturated oils under these conditions, while the TO showed only 19 meq O_2/kg oil of peroxide value and as much as 15 % polymers. In relation to the composition of volatile compounds, that found in the SO was close to that expected from the cleavage of the alkoxyl radicals formed from the LA-derived hydroperoxides, where hexanal is the main compound. However, the composition of volatile compounds of the TO was characterized by the occurrence of heptanal and *t*-2-nonenal, which were absent in the SO. An alternative route of formation for these distinct volatile oxidation compounds in TO could be scission of dioxoethanes coming from 1,2 cycloadditions of CLA with oxygen. Overall, the results obtained in this study, both on non-volatile and on volatile compounds, support that oxidation kinetics of CLA-rich oils differ substantially from that expected according to the hydroperoxide theory. Oxidation of CLA seems to proceed preferentially by the addition of the peroxyl radical to a double bond during propagation reactions, thus supporting the formation of oligomeric peroxides from the early events of lipid degradation.

Keywords CLA · Oxidation · Polymers · Volatiles

Introduction

Oils rich in conjugated linoleic acid (CLA), such as Tonalin® oil (TO), are functional ingredients nowadays added to a variety of foods due to their health-promoting effects [1–3]. CLA is a mixture of positional and geometric isomers of octadecadienoic acid with conjugated double bonds, and oils rich in CLA are normally obtained through alkaline isomerization of safflower oil (SO)—an oil rich in linoleic acid (LA) [4]. Commercial CLA-rich oils consist almost entirely of the two biologically active CLA isomers (9-*cis*, 11-*trans* and 10-*trans*, 12-*cis* linoleic acids) in approximately equal amounts (about 45 % each). In particular, dairy products with added CLA have nowadays great potential within the emerging nutraceutical and functional food markets [1].

Despite the growing consumption of CLA-rich oils, safety concerns regarding the use of CLA persist and specifically in relation to the formation of oxidized compounds that could lead to adverse physiological effects in cardiovascular and cancerous processes, which are precisely the same targets for potential health benefits of CLA [5, 6]. Little is known about oxidation kinetics of CLA and the main variables affecting oxidative stability of CLA-fortified or CLA-added foods. In fact, it is only generally agreed that oxidation pathways of CLA are unclear [6–13].

Measurement of oxidation in CLA-rich oils is carried out by a variety of methods that do not often indicate the

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real level of oxidation and provide contradictory results. Fatty acid composition is routinely used by manufacturers just to ensure that the labelled level of CLA in functional products is within the range claimed and the only oxidation parameter used as quality specification is the peroxide value. However, unlike the LA, hydroperoxides seem to be only minor compounds in the oxidation of CLA and polymers are formed from the very beginning of the oxidation process [8, 11, 13, 14]. Regarding measurement of CLA loss based on fatty acid analysis, it lacks of sufficient sensitivity to detect oxidation levels at initial stages [8, 11]. Yet, fatty acid composition and peroxide value determination remain as the most widely used methods to evaluate oxidation of CLA substrates in research studies [15–19]. As to volatile oxidation compounds, we published the first study in this context and found that CLA-rich oils showed a distinct composition and that heptanal could be used as a maker of their oxidation progress [20]. Other authors have recently confirmed these results for CLA in different chemical forms [21–23].

The objective of the present work was, for the first time to our knowledge, to monitor joint formation of non-volatile and volatile oxidation compounds in TO, a CLA-rich oil, as compared with its parent, LA-rich oil, SO. Oxidation conditions close to ambient, shelf-life storage were selected (40 °C in the dark). Analytical evaluations included identification and quantification of volatiles by solid-phase microextraction (SPME) and GC–FID/GC–MS and, in the case of non-volatile compounds, measurement of peroxide value (PV) and quantification of polymers by high-performance size-exclusion chromatography. Additionally, tocopherols were determined throughout the oxidation process.

Materials and methods

Materials

Tonalin® TG 80 oil (TO) was acquired from Cognis Nutrition & Health (Cincinnati, OH, USA), and refined SO was purchased from Interfat SA (Barcelona, Spain). Solid-phase microextraction (SPME) fibres coated with 75 μm carboxen™/polydimethylsiloxane (CAR/PDMS) were obtained from Supelco (Bellefonte, PA, USA). Tridecanoin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and bromobenzene from Fluka Chemika (Buchs, Switzerland). Palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (*9c*, *12c* 18:2), 9-*cis*, 11-*trans* linoleic acid (*9c*, *11t* 18:2) and 10-*trans*, 12-*cis* linoleic acid (*10t*, *12c* 18:2) were obtained from Nu-Chek-Prep, Inc. (Elysian, MN, USA). Other chemicals and reagents used were of analytical grade and obtained from local suppliers.

Oxidation assays

Triplicate samples of TO and SO (40 g each) were placed in 50-mL dark vials and stored in a temperature-controlled chamber at 40 ± 3 °C in the dark. Aliquots (1 g) were withdrawn for analyses along the oxidation experiment.

Methods

Analysis of fatty acid composition

Fatty acid composition was determined by GC–FID analysis. The oils were converted into fatty acid methyl esters using 2 M KOH in methanol [24]. FAME were analysed on an HP-6890 chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a split/splitless injector and a FID detector. Fatty acids were separated using an HP-Innowax capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Hewlett-Packard, Avondale, PA, USA). The column was held at 180 °C for 2 min after injection, temperature-programmed at 3 °C/min to 230 °C and held there for 20 min. A split ratio of 1:40 was applied, and hydrogen was used as carrier gas (1 mL/min). The injector temperature was set at 250 °C, and detector temperature was set at 270 °C. Pure palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (*9c*, *12c* 18:2), 9-*cis*, 11-*trans* linoleic acid (*9c*, *11t* 18:2) and 10-*trans*, 12-*cis* linoleic acid (*10t*, *12c* 18:2) from Nu-Chek-Prep were used as standards.

Determination of the peroxide value

Peroxide value was determined by the iodometric assay following IUPAC Standard Method 2.501 [24].

Determination of tocopherols

Tocopherols were determined by normal-phase HPLC with fluorescence detection according to IUPAC Standard Method 2.411 [24].

Quantitation of triacylglycerol polymers

Aliquots of 50 mg oil were dissolved in 1 mL tetrahydrofuran for direct analysis by HPSEC. A chromatograph equipped with a Rheodyne 7725i injector with a 10-μL sample loop, a Knauer 120 HPLC pump (Knauer, Berlin, Germany) and a Merck L-7490 refractive index detector (Merck, Darmstadt, Germany) was used. The separation was performed on two 100 and 500 Å Ultrastyragel columns (25 cm × 0.77 cm i.d.) packed with porous, highly cross-linked styrene–divinylbenzene copolymers (particle size: 5 μm) (Hewlett-Packard, Avondale, PA, USA) connected in series, with tetrahydrofuran (1 mL/min) as the

Table 1 Composition and oxidative parameters of safflower and Tonalin® oils

	Safflower oil	Tonalin oil
Fatty acid composition (wt%)		
16:0	7.2 ± 0.2	2.4 ± 0.1
18:0	2.6 ± 0.1	2.6 ± 0.1
18:1	13.7 ± 0.5	14.2 ± 0.6
18:2 *9c*, *12c*	74.7 ± 0.7	0.5 ± 0.1
18:2 *9c*, *11t* (CLA)	38.2 ± 0.7	
18:2 *10t*, *12c* (CLA)	38.6 ± 0.7	
Others	1.8 ± 0.1	3.5 ± 0.2
Tocopherols (mg/kg)		
α	266 ± 13	28 ± 2
γ		324 ± 17
δ		215 ± 13
Peroxide value (meq O_2/kg)	2.9 ± 0.2	2.5 ± 0.3
Polymers (%)	1.0 ± 0.3	1.1 ± 0.4

Data are expressed as means ± standard deviations ($n = 3$)

c: *cis*; *t*: *trans*; CLA: conjugated linoleic acid

mobile phase according to IUPAC Standard Method 2.508 [24]. The groups of compounds quantified were dimers and oligomers of triacylglycerols [25]. The sum of dimers and oligomers will be referred to as polymers.

Analysis of volatiles

Solid-phase microextraction (SPME) of volatiles was performed using a 75-µm carboxen/polydimethylsiloxane (CAR/PDMS) fibre mounted in a SPME manual holder assembly (Supelco, Poole, UK). The fibre was conditioned for 30 min in the injection port of the GC at 250 °C as recommended by the manufacturer. A 500-mg aliquot of sample was weighed in a 20-mL dark vial. Ten microlitres of internal standard solution (0.75 mg/mL of bromobenzene in methanol) and a stirring bar were added. The vial was capped with a PTFE septum (QMX Laboratories, Thaxted, UK) and placed in a water bath (40 °C) on a magnetic stirrer, and the sample was equilibrated for 5 min at the required temperature before SPME sampling. The septum was manually pierced with the SPME needle, and the fibre was exposed to the sample headspace for 30 min. Volatiles were analysed with a Hewlett-Packard (Palo Alto, CA, USA) 5890 series II gas chromatograph equipped with a FID detector, a split/splitless injector and a CP–Sil 8 CB low bleed/MS fused-silica capillary column (5 % phenyl/95 % PDMS; 60 m × 0.25 mm i.d., 0.25 µm film thickness; Varian-Chrompack). A section of column nearest to the injection port was cooled in a beaker of powdered solid carbon dioxide to cryofocus the volatiles. Volatile compounds were desorbed from the SPME fibre onto the front of column for 3 min. The injection port was in splitless mode, and split flow was programmed to turn on after 0.5 min. The temperature of the injector was 250 °C. After fibre desorption, the solid carbon dioxide was removed and the GC programme started. The oven was maintained at 40 °C for a further 2 min, and then, the temperature was raised to 120 °C at 4 °C/min and then at 20 °C/min to 250 °C, and held at 250 °C for 10 min. The FID temperature was 280 °C. Helium at 16 psi was used as the carrier gas. *n*-Alkanes (C_5–C_{25}) were run under the same conditions to obtain linear retention index (LRI) values for the components [26]. The identification of compounds was performed on a Hewlett-Packard (Palo Alto, CA, USA) 5972 mass spectrometer, coupled to a 5890 series II gas chromatograph and a G1034C Chemstation. The mass spectrometer was operated in electron impact mode with an electron energy of 70 eV and an emission current of 50 µA. The ion source was maintained at 170 °C. The mass spectrometer scanned from *m/z* 29 to *m/z* 400 at 1.9 scans/s. Compounds were identified by first comparing their mass spectra with those contained in the National Institute of Standards and Technology (NIST) followed by comparing LRI values with *n*-alkanes (C_5–C_{25}) [26].

Statistical analysis

Data of fatty acid composition, tocopherols, peroxide value and polymers of the starting oils were obtained by using three determinations. Data reported for the oxidation experiments were obtained from triplicate samples and expressed as mean values and SDs. Excel 2000 (Microsoft Corporation, Redmond, WA, USA) was used for data analyses.

Results and discussion

Characterization of oils

Table 1 shows fatty acid and tocopherol compositions and oxidative parameters of SO and TO. The content of linoleic acid (C18:2 9*c*, 12*c*) in the SO was similar to that of total CLA (C18:2 9*c*, 11*t* and C18:2 10*t*, 12*c*) in the TO. However, the TO contained approximately twice as much of total tocopherols as the SO, and γ and δ homologues were the most abundant tocopherols. The PV and initial polymer content were low in both oils and typical for fresh refined oils.

Changes in PV, tocopherols and polymers during oxidation

Figure 1 shows changes of PV and tocopherols during oxidation of the SO and TO at 40 °C. As expected, the

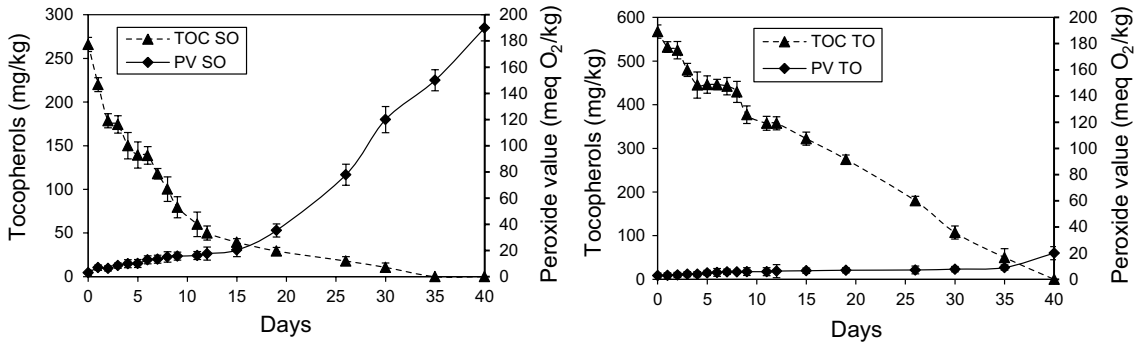

Fig. 1 Peroxide values (PV) and tocopherols content (TOC) of safflower oil (SO) and Tonalin® oil (TO) during storage at 40 °C in the dark. *Error bars* indicate standard deviations of the means ($n = 3$)

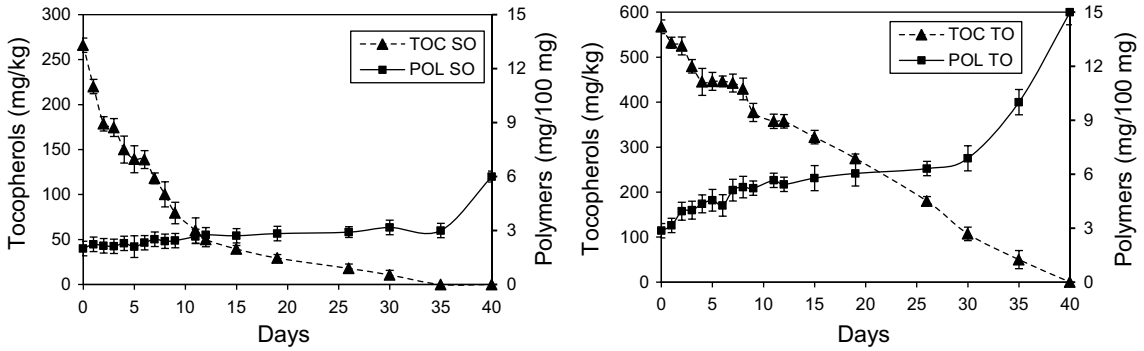

Fig. 2 Polymer concentrations (POL) and tocopherols content (TOC) of safflower oil (SO) and Tonalin® oil (TO) during storage at 40 °C in the dark. *Error bars* indicate standard deviations of the means ($n = 3$)

SO showed a progressive rise in the PV from the beginning and increased markedly once tocopherols were only present in very low amounts. At the end of the induction period, 35 days, when tocopherols were exhausted, PV was 152 meq O_2/kg. In sharp contrast, the PV did not increase significantly during the induction period in the TO, and at 40 days, when tocopherols were exhausted, the PV was only 19 meq O_2/kg.

Figure 2 shows changes in polymers together with loss of tocopherols during oxidation of the SO and TO at 40 °C. In the SO, polymers practically remained constant and below 3 % till the end of the induction period, and in fact, a significant rise in polymers marked the onset of advanced oxidation. This oxidation pattern has been repeatedly observed in our previous studies on oils and model triacylglycerols [27–29]. Nevertheless, the TO showed a very different oxidation pattern since polymers started to increase from the beginning, reaching amounts as high as 15 % at the point of tocopherols exhaustion.

The results obtained in the present work agree with those we previously found in oxidation studies on conjugated and non-conjugated methyl linoleate [11] and those reported by Suzuki et al. [14] on conjugated linolenic acid-rich oil (bitter gourd oil) and non-conjugated linolenic acid-rich oil (soybean oil). It is well known that the mechanism of autoxidation of methylene-interrupted fatty acid double bonds involves a catalytic process which proceeds via a free radical mechanism [30]. The initiation step consists in alkyl radical formation by the abstraction of a hydrogen radical from the carbon adjacent to the double bond. In the propagation step, oxygen is added to form alkylperoxyl radicals and the oxygen consumed is primarily converted to hydroperoxides. However, the low occurrence of hydroperoxides and high polymerization observed for the TO in the present work support that different mechanisms could be involved. In this regard, pioneering studies on conjugated polyunsaturated fatty acids already established that oxygen-containing polymers were main oxidation products [31], and suggested that carbon-to-oxygen polymerization occurred rather than carbon-to-carbon polymerization [32] and that the peroxide decomposition is not a major factor in the mechanism of oxidation of conjugated substances [33].

Figure 3 shows the HPSEC chromatograms obtained at 35 days for the SO and TO. At that point, close to the end of the induction period for both oils, the amounts of polymers were markedly different, 3.1 versus 15 %, respectively.

Moreover, it is especially noticeable that, while polymers were essentially dimers in the SO, polymers were constituted almost exclusively by trimmers and higher oligomers in the TO. This finding was observed from the beginning of the oxidation progress. These results are consistent with those we previously obtained in conjugated methyl linoleate, which showed for the first time the large degree of polymerization (higher than three) occurring from the beginning of the oxidation process under mild storage conditions [11]. In this regard, in studies inducing polymerization with Co/Ca/Zr drier and using various mass spectrometry techniques, Muizebelt and Nielen [34] reported formation of up to hexamers in a conjugated fatty acid ester and SIMS traces indicated that up to 15 oxygens were incorporated in the hexamer group. Apart from the contribution of oxygenated functional groups in fatty acyls, the large number of oxygens observed with SIMS in oligomers suggests abundance of peroxyl cross-links.

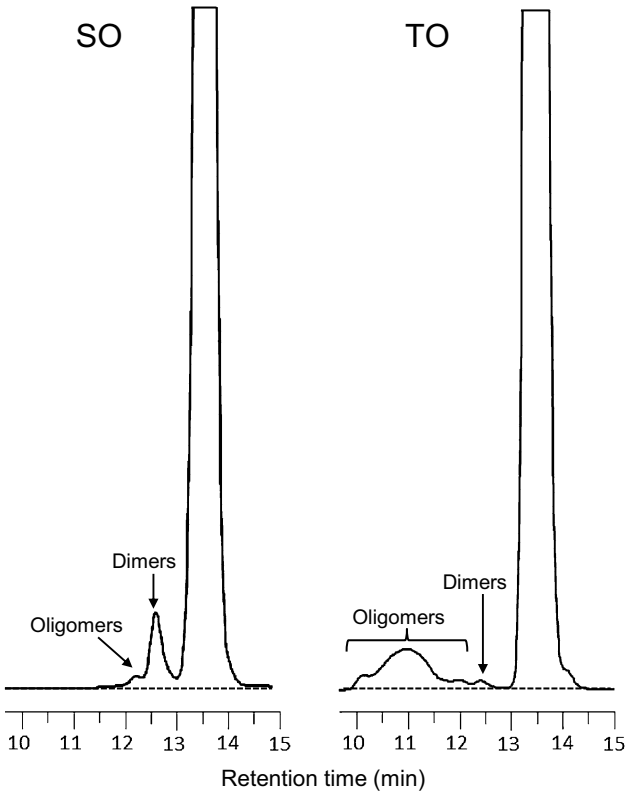

Fig. 3 Elution profiles by HPSEC of safflower oil (SO) and Tonalin® oil (TO) after 35 days at 40 °C in the dark

Figure 4 shows a schematic representation which helps illustrate the different results found for the SO and TO, showing formation of hydroperoxides from LA in SO triacylglycerols and formation of oligomeric peroxides from CLA in TO triacylglycerols. Differences found could be explained in terms of bond dissociation energy (BDE) as suggested by Oyman et al. [35]. For LA oxidation, the most favourable reaction of the peroxyl radical formed in the propagation step is the H-abstraction from the double allylic group of LA (BDE: 272 kJ/mol) to yield hydroperoxides as primary oxidation products. However, in CLA the preferred reaction pathway for the peroxyl radical formed is the addition to a conjugated double bond (BDE: 284 kJ/mol) rather than the abstraction of a monoallylic H atom (BDE: 322 kJ/mol). Also, in CLA, addition of the peroxyl radical to the conjugated diene system leads to formation

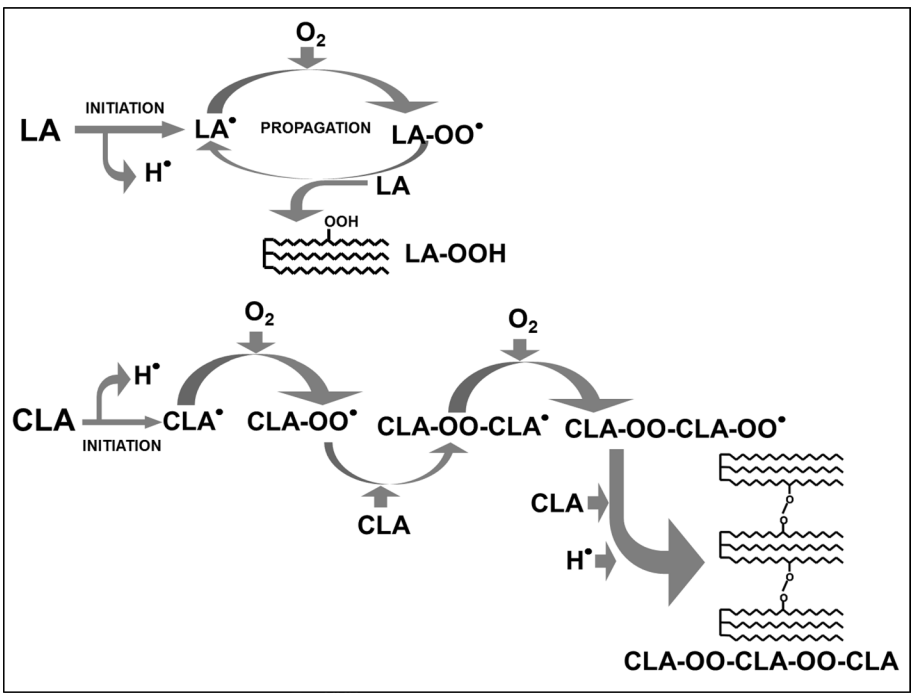

Fig. 4 Schematic representation of formation of hydroperoxides from linoleic acid (LA) and oligomeric peroxides from conjugated linoleic acid (CLA) in triacylglycerols of safflower oil (SO) and Tonalin® oil (TO), respectively

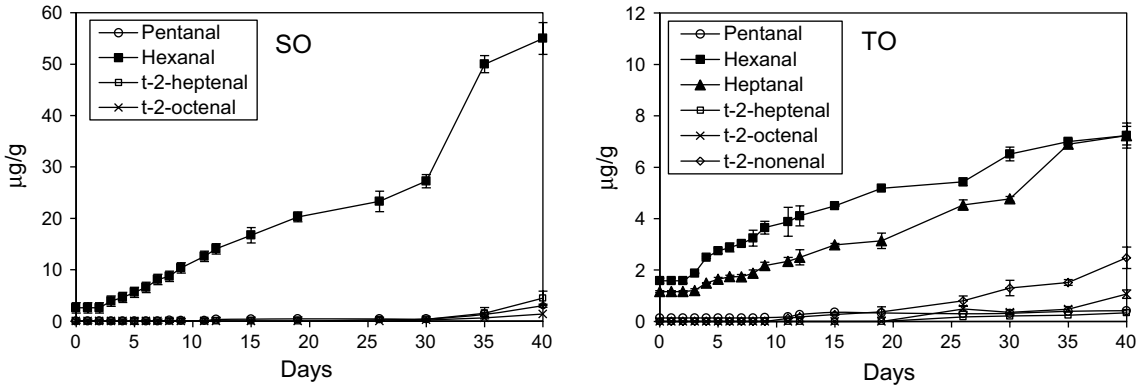

Fig. 5 Main volatiles oxidation compounds formed during storage at 40 °C in the dark. *Error bars* indicate standard deviations of the means ($n = 3$). *SO* safflower oil, *TO* Tonalin® oil

of resonance-stabilized allylic radical intermediates. These differences in oxidation mechanisms would account for the low hydroperoxide amounts (PV) formed throughout the oxidation of TO in favour of formation of peroxyl radical dimers leading ultimately to oligomeric peroxides. Such polymers cannot be therefore considered termination products characteristic of advanced oxidation stages, but primary oxidation compounds formed during the propagation stage.

Formation of volatile compounds during oxidation

Figure 5 shows time course of formation of the main volatile compounds in the SO and TO. In the SO, the volatile profile was close to that expected from the cleavage of the alkoxyl radicals formed from the hydroperoxides of autoxidized LA, being hexanal the main volatile oxidation product. Pentanal, *t*-2-heptanal and *t*-2-octenal were other major volatiles formed during oxidation of the SO and also produced by decomposition of the most abundant LA hydroperoxides [30]. Major volatiles found in the TO were not those expected from theoretically stable hydroperoxides formed in CLA, as we already reported in samples oxidized at 60 °C [20]. The composition of volatile compounds in oxidized TO was characterized by the joint occurrence of two major volatile oxidation products, i.e. hexanal and heptanal, from which the latter compound was absent in the oxidized SO. Another important difference was the presence of *t*-2-nonenal in TO, while it was totally absent in the SO. According to the hydroperoxide theory, formation of hexanal and pentanal is predictable from the expected major 13-hydroperoxides formed in both 9*c*, 11*t*-CLA and 10*t*, 12*c*-CLA, both isomers present in equal amounts in TO. In contrast, the presence of heptanal and *t*-2-nonenal, as well as other minor unidentified compounds, is not easily explained. Heptanal could come from β-scission of the alkoxyl radical formed from 12-hydroperoxy-8-*t*,10-*t*-octadecadienoate, which has been reported to be one of the hydroperoxides formed in oxidized 9-*c*, 11-*t* CLA [35]. However, *t*-2-nonenal would be a product derived from the 10-hydroperoxy-8-*t*,11-*t*-octadecadienoate or 10-hydroperoxy-8-*t*,11-*c*-octadecadienoate in oxidized 9-*c*, 11-*t* CLA. However, such hydroperoxides are unlikely to be formed because of the instability of the resonance structures since the double bonds are not conjugated in the pentadienyl radicals [36]. Likewise, *t*-2-octenal would come from the supposedly unexpected 11-hydroperoxy-9,12-octadecadienic fatty acid [37, 38] from both 9-*c*, 11-*t* CLA and 10-*t*, 12-*c* CLA, which would also contribute to formation of further amounts of heptanal. An alternative route of formation for the oxidation volatile compounds found in the TO could be based on the mechanisms proposed by Yurawecz and co-workers. They suggested that CLA may undergo 1,2 cycloadditions with oxygen resulting in dioxoethanes that would lead to volatile formation and proposed that heptanal would be a scission product of a 11,12-dioxoethane, whereas 2-nonenal would result from the scissions of 9,10-dioxoethane [9].

It is also of great relevance that much higher amounts of oxidation volatiles were formed in the SO than in the TO. This could be explained as the result of the strong buildup of hydroperoxides in the SO leading to higher amount of volatiles via β-scission in contrast to the predominant route of formation of oligomeric peroxides in the case of SO (Fig. 4). In view of these results, it is not strange that sensory analyses of CLA-enriched products had not reported increased oxidized flavour [6, 39].

Conclusions

The results obtained in this study show that different oxidation mechanisms are involved in LA-rich and CLA-rich oils. Polymers formation and not hydroperoxides formation

occurred in the TO from the beginning of the oxidation process, thus invalidating peroxide value as oxidation measurement for CLA-rich oils. This is of utmost importance since, at present, PV is the only determination included in quality specifications of commercialized CLA-rich oils. Results on volatile formation showed a markedly different pattern for TO compared to SO, being heptanal distinctive of the oxidation progress of TO. Since volatile patterns of TO and SO greatly differed qualitatively and quantitatively, it is necessary to reconsider the sensory analyses currently used as a tool to detect oxidation of functional food products with CLA-rich oils. Overall results showed that polymer formation is an early event of the oxidation process of CLA-rich oils and hence the most appropriate method to control oxidation in CLA-rich oils.

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Compliance with ethical standards

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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3.8.- Efectividad del α-, γ- y δ-tocoferol en un aceite rico en CLA.

Effectiveness of α-, γ- and δ-tocopherol in a CLA-rich oil.

Gloria Márquez-Ruiz, María del Carmen García-Martínez, Francisca Holgado y Joaquín Velasco.

Antioxidants, 2014; 3: 176-188.

Resumen:

El ácido linoleico conjugado (CLA) es una mezcla de isómeros posicionales y geométricos del ácido octadecadienoico con dobles enlaces conjugados. Se le han atribuido diferentes propiedades beneficiosas para la salud a algunos isómeros, como actividad anticarcinogénica, efectos antiescleróticos y la reducción de grasa corporal. Por tanto, actualmente se utilizan en alimentos funcionales aceites ricos en CLA como el aceite Tonalin® (TO), normalmente obtenido a través de la isomerización alcalina del aceite de cártamo (SO), un aceite rico en ácido linoleico (LA). Sin embargo, es esencial proteger estos aceites de la oxidación para asegurar la calidad de los alimentos suplementados. El objetivo de este trabajo fue evaluar la oxidación y la efectividad de diferentes homólogos del tocoferol (α-, γ- y δ-), solos o combinados con sinergistas (palmitato de ascorbilo y lecitina), en TO y SO en distintas condiciones, es decir, temperatura ambiente (25ºC) y condiciones aceleradas en Rancimat (100ºC). Se analizaron los aceites originales y los aceites con los antioxidantes seleccionados adicionados y tras eliminar previamente sus antioxidantes naturales. Los resultados mostraron grandes diferencias entre SO y TO en términos de formación de hidroperóxidos y polímeros, así como también en la efectividad de los tocoferoles para retrasar la oxidación. El TO mostró niveles superiores de polimerización y, en general, la efectividad de los homólogos del tocoferol, solos o en combinación con sinergistas, también fue menor en el TO.

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Article

Effectiveness of α-, γ- and δ-Tocopherol in a CLA-Rich Oil

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Abstract: Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of octadecadienoic acid with conjugated double bounds. Positive health properties have been attributed to some isomers, such as anticarcinogenic activity, antiartherosclerotic effects and reduction of body fat gain. Hence, oils rich in CLA such as Tonalin® oil (TO), normally obtained through alkaline isomerization of safflower oil (SO), an oil rich in linoleic acid (LA), are currently used in functional foods. However, special care must be taken to protect them from oxidation to ensure the quality of the supplemented foods. The objective of this work was to evaluate the oxidation and effectiveness of different tocopherol homologues (α-, γ- and δ-), alone or in combination with synergists (ascorbyl palmitate and lecithin), in TO compared to SO at different conditions, ambient temperature (25 °C) and accelerated conditions in Rancimat (100 °C). The oils, the oils devoid of their antioxidants and the latter containing the antioxidants added were assayed. Results showed great differences between SO and TO in terms of formation of hydroperoxides and polymers and also in the effectiveness of tocopherols to delay oxidation. TO showed higher levels of polymerization and, in general, the effectiveness of tocopherol homologues, alone or in combination with synergists, was also lower in the TO.

Keywords: antioxidants; CLA; conjugated linoleic acid; functional lipids; lipid oxidation; peroxide value; polymers; tocopherols

1. Introduction

Oils rich in conjugated linoleic acid (CLA) are nowadays functional ingredients that are added to a variety of foods due to their potentially beneficial health effects. CLA acronym includes a group of positional and geometric isomers of linoleic acid. Numerous animal studies indicate that CLA, specifically the *cis*-9, *trans*-11 isomer, may influence diverse physiological functions and promote health with regard to cancer, atherosclerosis, bone formation, growth modulation and immunity [1]. CLA-rich oils are obtained mainly from alkaline isomerization of linoleic acid-rich oils such as sunflower, soybean and safflower oils [2], since CLA isomers occur naturally only in very minor amounts in food from ruminants, milk and meat. Commercial CLA-rich oils consist almost entirely (*i.e.*, >80%) of the two biologically active CLA isomers (*cis*-9, *trans*-11 and *cis*-10, *trans*-12 linoleic acids) in approximately equal amounts (about 45% each). Although there is no evidence that consumption of such CLA preparations could induce adverse effects in healthy humans [3], safety concerns regarding the use of CLA persist and need further investigation [4].

Despite the growing interest and consumption of CLA-rich oils, scarce information is available on their oxidative stability and effectiveness of antioxidants [5]. However, it is essential to gain insight into the occurrence of oxidised compounds in commercial CLA preparations, since they could lead to adverse physiological effects in cardiovascular and cancerous processes [6], which are precisely the same targets for potential health benefits of CLA.

Studies on CLA oxidation are often contradictory. Oxidation pathways of CLA are still unclear and the mechanisms involved remain controversial [7–14]. Some authors [7,8,11–13] have suggested that, in contrast to the case of linoleate, hydroperoxides are only minor products in the oxidation of conjugated linoleate. Therefore, the methods normally used to control lipid oxidation in foods, e.g., peroxide value and thiobarbituric acid reactive substances, may not indicate the real level of oxidation [15,16] and provide misleading results when comparing oxidative stability of CLA and LA. This could be in great part due to formation of polymers from the very beginning of the oxidation process [7,11].

Only three studies have been found in relation to the effect of antioxidants in CLA-rich oils. Effectiveness of tocopherol homologues (α-, β-, γ- and δ-tocopherol) has been studied in CLA at 50 °C through analyses of peroxide value and TBARS, concluding that δ- and γ-tocopherol exhibited the highest activity, whereas α-tocopherol had the lowest protective effect [17]. Likewise, as compared to the synthetic antioxidants TBHQ, BHA and BHT and also to gallic acid, α-tocopherol rendered lower protection in CLA at 45 °C [18]. To our knowledge, the only published work focused on CLA-rich oils tested α-tocopherol in an oil devoid of antioxidants and concluded that it increased its stability to a level similar to that of the LA-rich oil [16]. In this case, results were obtained through determination of the remaining unoxidised substrate and TBARS. The authors found that loss of substrate in CLA-rich oils was significantly greater than that expected from the data obtained for TBARS. This finding could be accounted for by the formation of polymers, as we have already reported in kinetic studies on oxidation of methyl conjugated linoleate [11].

The objective of this work was to evaluate the oxidation and effectiveness of different tocopherol homologues, alone or in combination with synergists (ascorbyl palmitate and lecithin), in a CLA-rich oil, *i.e.*, Tonalin® oil (TO). Different conditions, ambient temperature (25 °C) and accelerated

conditions in Rancimat (100 °C), were applied. For comparative purposes, a LA-rich oil, *i.e.*, safflower oil (SO), was also studied. Formation of primary oxidation products, through peroxide value determination, and advanced oxidation products, through quantification of polymers, was monitored in all assays. The intact oils, the oils devoid of their antioxidants and the latter containing α-, γ- or δ-tocopherol added, alone or in combination with both ascorbyl palmitate and lecithin, were studied.

2. Experimental Section

2.1. Materials and Samples

Tonalin® TG 80 oil (TO) was acquired from Cognis Nutrition & Health (Cincinnati, OH, USA), and refined safflower oil (SO) was purchased from Interfat S.A. (Barcelona, Spain). TO and SO were passed over aluminum oxide (activated at 200 °C for 3 h) column twice in order to remove tocopherols and minor polar compounds as described by Yoshida and coworkers [19]. The tocopherols (α-, γ- and δ-) (purity ≥ 99%), ascorbyl palmitate and lecithin (APL) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). α-, γ- or δ- Tocopherol (500 mg/kg), alone or in combination with ascorbyl palmitate (300 mg/kg) and lecithin (5000 mg/kg), were added to the oils stripped of their naturally occurring antioxidants. Chemicals and reagents used were of analytical grade and obtained from local suppliers.

2.2. Oxidation Procedures

Assays under Rancimat conditions: Oil Stability Index (OSI) of TO and SO (starting oils, oils devoid of antioxidants and the latter containing tocopherols added, alone or with APL) was obtained in a Rancimat device (Metrohm, Herisau, Switzerland) at 100 °C with 20 L/h air flow using samples of 2.5 ± 0.1 g [20]. The OSI values thus obtained, as well as the conductivity reading provided by the device, were used in subsequent experiments to carry out appropriate samplings for satisfactory follow-up of oxidation along the test. Special attention was paid to take samples at the end of the induction period (IP), *i.e.*, at times equal to the OSI point. Experiments were done in triplicate. In samples containing antioxidants added, the protection factor was calculated as the ratio between the OSI values in the presence and absence of antioxidants.

Assays at ambient temperature: Five-gram samples of TO and SO (starting oils and oils devoid of antioxidants) were placed in 1 L-glass beakers. The beakers were placed at a temperature-controlled chamber and stored at 25 ± 3 °C in the dark. Aliquots (100 mg) were withdrawn for analyses along the oxidation experiment. Experiments were done in triplicate.

2.3. Analytical Methods

2.3.1. Determination of Tocopherols

Tocopherols were determined by normal-phase HPLC with fluorescence detection according to IUPAC Standard Method 2.411 [21].

2.3.2. Quantitation of Triacylglycerol Dimers and Higher Oligomers by High-Performance Size-Exclusion Chromatography (HPSEC)

Aliquots of 50 mg oil were dissolved in 1 mL tetrahydrofuran for direct analysis by HPSEC. A chromatograph equipped with a Rheodyne 7725i injector (Hamilton, NV, USA) with a 10-μL sample loop, a Knauer 120 HPLC pump (Knauer, Berlin, Germany) and a Merck L-7490 refractive index detector (Merck, Darmstadt, Germany) was used. The separation was performed on two 100 and 500 Å Ultrastyragel columns (25 cm × 0.77 cm ID, Hewlett-Packard, Avondale, PA, USA) packed with porous, highly cross linked styrene-divinylbenzene copolymers (particle size: 5 μm) (Hewlett-Packard, Avondale, PA, USA) connected in series, with tetrahydrofuran (1 mL/min) as the mobile phase according to IUPAC Standard Method 2.508 [21]. The groups of compounds quantified were dimers and higher oligomers [22]. The sum of dimers and higher oligomers will be referred to as polymers.

2.3.3. Determination of the Peroxide Value (PV)

Peroxide value was determined by the iodometric assay following IUPAC standard method 2.501 [21].

2.3.4. Analysis of Fatty Acid Composition

Fatty acid composition was determined by GC-FID analysis. The oils were converted into fatty acid methyl esters (FAME) using 2 M KOH in methanol (IUPAC, 1992). FAME were analysed on an HP-6890 chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with a split/splitless injector and a FID detector. Fatty acids were separated using an HP Innowax capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness, Hewlett Packard, Avondale, PA, USA). The column was held at 180 °C for 2 min after injection, temperature-programmed at 3 °C/min to 230 °C and held there for 20 min. A split ratio of 1:40 was applied and hydrogen was used as carrier gas (1 mL/min). The injector temperature was set at 250 °C and detector temperature was set at 270 °C.

2.4. Statistical Analysis

Data of fatty acid composition, tocopherols, peroxide value, polymers and OSI in the starting oils, as well as OSI in the oils devoid of their antioxidants and with tocopherols and synergists added, were obtained by using three determinations. The oxidation experiments were carried out in triplicate and results for polymers and peroxide value have been expressed as mean values. Comparisons between means were made by applying one-way ANOVA using SPSS Statistics version 17.0 (SPSS Inc., Dublin, Ireland). Differences between means were determined using post-hoc Tukey's test. Significant differences were established at $p < 0.05$.

3. Results and Discussion

Table 1 shows fatty acid and tocopherol compositions and oxidative parameters of SO and TO. The content of linoleic acid (C18:2 9c, 12c) in SO was similar to that of total CLA (C18:2 9c, 11t and C18:2 10t, 12c) in TO. Even though TO contained approximately twice as much of total tocopherols as

SO, the two oils presented similar OSI values, 5.8 and 5.7 h for SO and TO, respectively. The PV and polymers were low in both oils and typical for fresh refined oils.

Table 1. Composition and oxidative parameters of Safflower and Tonalin® oils.

Parameter	Safflower Oil	Tonalin Oil
Fatty acid composition (%)		
16:0	7.2 ± 0.2	2.4 ± 0.1
18:0	2.6 ± 0.1	2.6 ± 0.1
18:1	13.7 ± 0.5	14.2 ± 0.6
18:2 *9c*, *12c*	74.7 ± 0.7	0.5 ± 0.1
18:2 *9c*, *11t* (CLA)		38.2 ± 0.7
18:2 *10t*, *12c* (CLA)		38.6 ± 0.7
Others	1.8 ± 0.1	3.5 ± 0.2
Tocopherols (mg/kg)		
α	266 ± 13	28 ± 2
γ		324 ± 17
δ		215 ± 13
Peroxide value (meq O_2/kg)	2.9 ± 0.2	2.5 ± 0.3
Oil Stability Index (h)	5.8 ± 0.4	5.7 ± 0.3
Polymers (%)	1.0 ± 0.3	1.1 ± 0.4

Data are expressed as Means ± Standard Deviations (n = 3). SO: safflower oil. TO: Tonalin® oil; *c*: *cis*; *t*: *trans*; CLA: conjugated linoleic acid.

Figure 1 shows the progress of different parameters during oxidation in Rancimat at 100 °C. In both oils, the end of the induction period or OSI value (dotted lines) was marked by the exhaustion of tocopherols (Figure 1A,B). At that point, the accelerated oxidation stage starts and a rapid increase in conductivity is normally obtained [23]. However, TO showed a slower increase in conductivity after the end of the induction period, thus indicating slower formation of volatile compounds detected by conductivity changes.

The most relevant differences between the oils were observed in the progress of PV and polymers. As expected, SO showed a progressive increase in the PV from the onset of the assay, reaching a value of 421 meq O_2/kg at the end of the induction period. Until then, the polymer content had not practically increased (2.1%), but exhaustion of tocopherols triggered polymerization beyond that point. Quite in contrast, TO showed the opposite oxidative behaviour, *i.e.*, a marked increase in polymers during the induction period, reaching values as high as 32.9% at the end and comparatively low PV levels (56 meq O_2/kg). The results obtained at 100 °C indicate that polymerization is favoured in TO at early stages of oxidation and tocopherols were not capable of preventing it.

With the aim of comparing SO and TO under realistic storage conditions, assays were carried out at 25 °C in the dark. Tables 2 and 3, and Figure 2 include the results obtained.

Figure 1. Oxidation of Safflower (**A**,**C**) and Tonalin (**B**,**D**) oils at 100 °C in Rancimat. Data are expressed as Means (n = 3 experiments). Coefficients of variation were equal to or lower than 8% for peroxide values and tocopherols, and 5% for polymers. PV: peroxide value, POL: polymers, Toc: tocopherols.

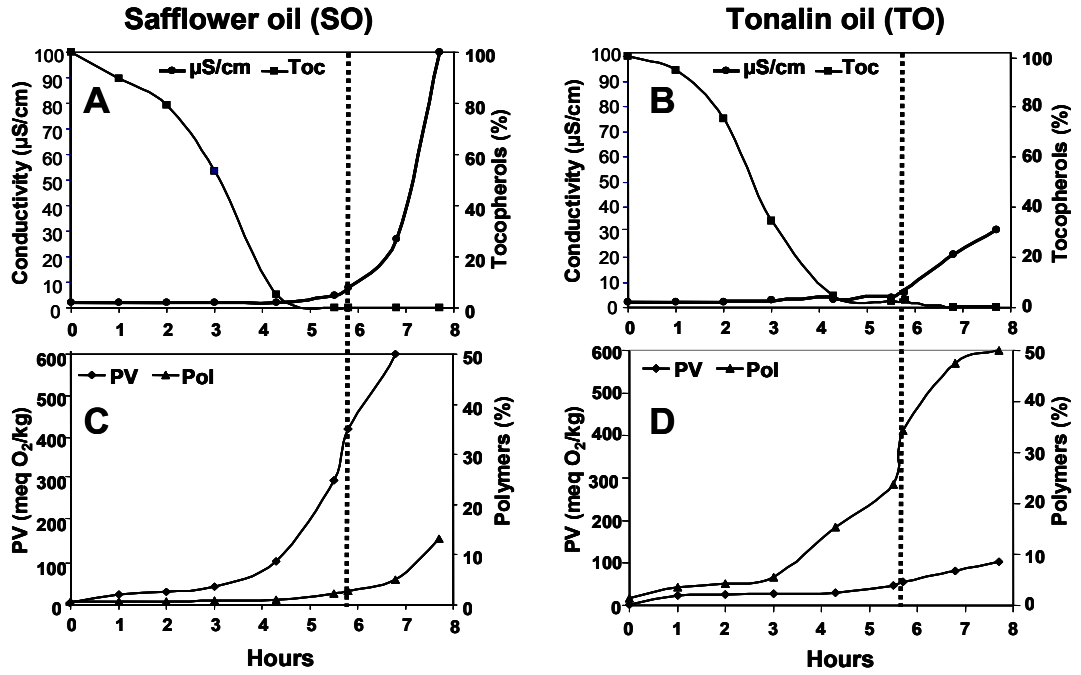


Table 2. Oxidation of safflower oil at 25 °C.

Days	Dimers (%)	Oligomers (%)	Total Polymers (%)	Peroxide Value (meq O_2/kg)	Tocopherols (mg/kg)
0	1.0	0.0	1.0	2.9	266
4	2.0	0.2	2.2	6.9	192
8	1.9	0.2	2.2	6.3	179
12	1.9	0.2	2.1	8.4	174
16	2.1	0.2	2.3	9.9	150
20	1.9	0.2	2.1	10.0	139
24	2.0	0.3	2.3	12.8	139
28	2.1	0.4	2.5	13.3	118
32	2.1	0.3	2.4	15.0	100
36	2.1	0.3	2.4	15.7	80
44	2.3	0.4	2.7	16.2	42
48	2.3	0.4	2.8	17.6	40
60	2.3	0.4	2.7	20.3	33
76	2.4	0.4	2.8	35.3	29
104	2.5	0.4	2.9	77.9	18
120	2.7	0.5	3.2	120.0	11
140	5.9	2.1	8.0	170.0	0

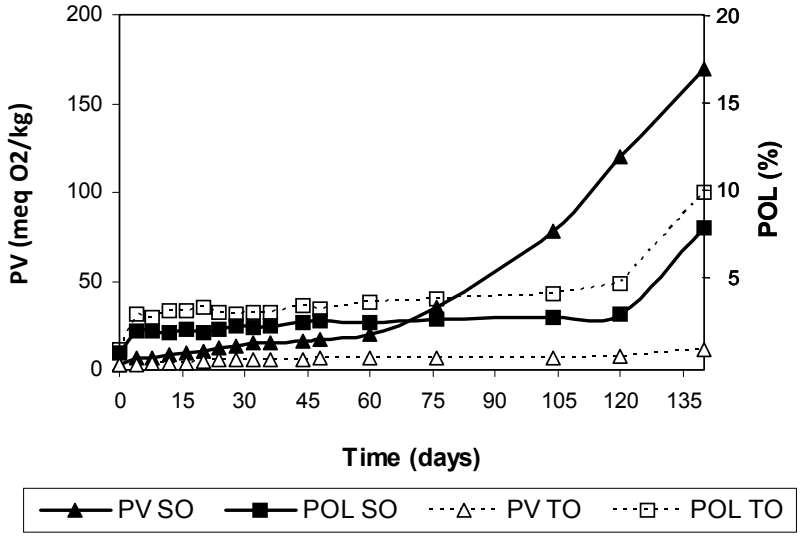
Data are expressed as Means (n = 3 experiments). Coefficients of variation were equal to or lower than 8% for peroxide values and tocopherols, and 5% for dimers, oligomers and total polymers.

Table 3. Oxidation of Tonalin oil at 25 °C.

Days	Dimers (%)	Oligomers (%)	Total Polymers (%)	Peroxide Value (meq O_2/kg)	Tocopherols (mg/kg)
0	0.0	1.1	1.1	2.5	568
4	0.2	2.9	3.2	2.9	532
8	0.2	2.7	2.9	3.3	525
12	0.2	3.1	3.4	4.0	480
16	0.2	3.1	3.4	4.0	445
20	0.3	3.3	3.6	4.9	446
24	0.2	3.0	3.3	5.3	446
28	0.2	2.9	3.1	5.7	442
32	0.2	3.1	3.3	5.6	429
36	0.2	3.0	3.2	5.9	377
44	0.2	3.4	3.7	5.8	358
48	0.2	3.3	3.4	6.3	357
60	0.2	3.6	3.8	6.5	322
76	0.2	3.8	4.0	6.9	275
104	0.2	4.1	4.3	7.1	180
120	1.6	3.3	4.9	7.8	107
140	1.8	7.2	10.0	11.0	0

Data are expressed as Means (n = 3 experiments). Coefficients of variation were equal to or lower than 8% for peroxide values and tocopherols, and 5% for dimers, oligomers and total polymers.

Figure 2. Peroxide values and polymer concentrations during storage of safflower oil (SO) and Tonalin oil (TO) at 25 °C in the dark. Data are expressed as Means (n = 3 experiments). Coefficients of variation were equal to or lower than 8% for peroxide values and 5% for polymers. PV: peroxide value, POL: polymers.



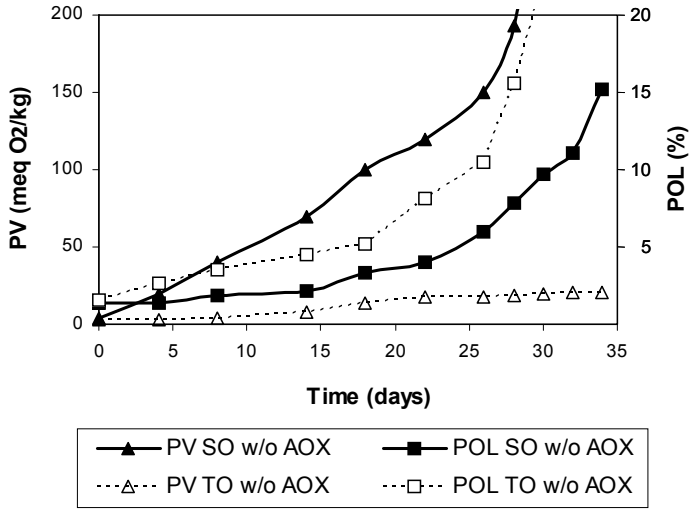
The end of the induction period in SO was around 120 days, as defined by the start of significant polymerization, PV above 100 meq O_2/kg and only negligible amounts of tocopherols remaining. This oxidation pattern has been repeatedly observed in our previous studies on oils and model triacylglycerols [24–26]. However, the induction period was far from clear in the case of TO.

Following also a total depletion of tocopherols after 120 days of storage, polymers increased more rapidly although their formation had started from the beginning of the assay, while PV changes were small and a level as low as 11 meq O_2/kg was reached at the end of the experiment.

The pool of polymers in TO comprised trimers and larger oligomers only (Table 3). In fact, the increase in polymers in TO throughout oxidation was essentially attributable to the increase of large oligomers. We already found a large degree of polymerization in a previous work comparing oxidation kinetics of conjugated methyl linoleate and non-conjugated methyl linoleate under mild oxidation conditions [11]. Similarly to the results obtained here in CLA-rich oils, conjugated methyl linoleate showed a very different oxidation pattern from that of non-conjugated methyl linoleate. Firstly, formation of typical primary oxidation products, *i.e.*, hydroperoxides, seemed to be negligible, secondly, dimer formation was rare and, thirdly, the starting point of oxidation was characterized by the appearance of polymers of unusually high molecular weight or size. These results indicate that formation of very complex molecules is favored even under the mild oxidation conditions used, *i.e.*, 25 °C in the dark.

For comparative purposes, the antioxidant-stripped oils were also tested at 25 °C. Clearly, polymer formation was greater in TO than in SO along oxidation and marked differences were observed in the PV (Figure 3). For example, the PV was over 200 meq O_2/kg in SO at 30 days of storage, while it was only 19 meq O_2/kg in TO.

Figure 3. Peroxide values and polymer concentrations during storage of antioxidant-stripped safflower oil (SO w/o AOX) and Tonalin oil (TO w/o AOX) at 25 °C in the dark. Data are expressed as Means (n = 3 experiments). Coefficients of variation were equal to or lower than 8% for peroxide values and 5% for polymers. PV: peroxide value, POL: polymers.



It is well known that the mechanism of autoxidation of methylene-interrupted fatty acid double bonds involves a catalytic process which proceeds via a free radical mechanism [27]. The initiation step consists of alkyl radical formation by the abstraction of a hydrogen radical from the carbon adjacent to the double bond. In the propagation step, oxygen is added to form alkylperoxyl radicals and the oxygen consumed is primarily converted to hydroperoxides. However, the low occurrence of hydroperoxides and high polymerization previously observed in CLA model compounds [11] and in

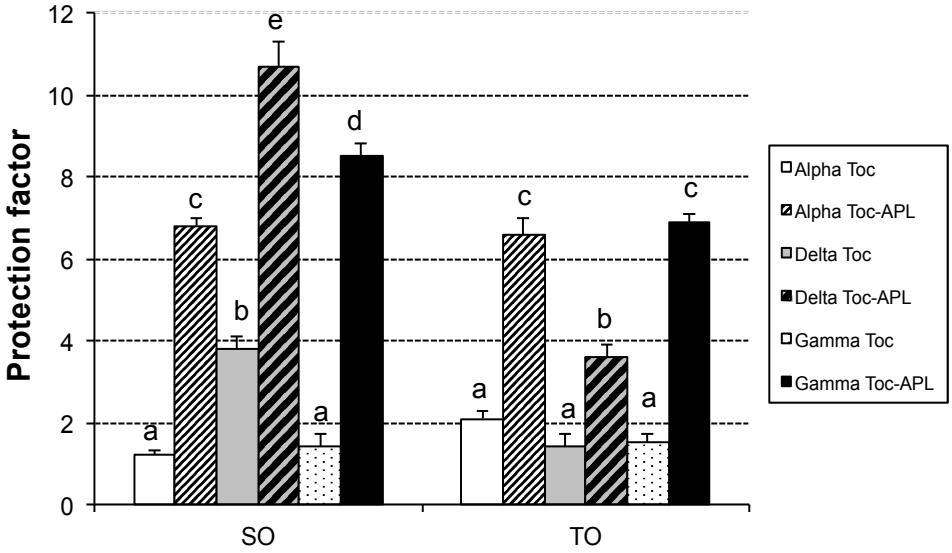
CLA-rich oils in the present work support that different mechanisms could be involved. Yurawecz and coworkers [8–12] suggested that the free radical mechanism is not likely to occur in conjugated fatty acids because high energy is previously required to separate double bonds from conjugation. Therefore, formation of oxidation compounds other than those expected from the free radical mechanism may be favoured. The authors reported that CLA underwent 1,2- and 1,4-cycloadditions with oxygen, which gave rise to dioxetane structures and endoperoxides leading to furan fatty acids, respectively. Other reactions such as dimerization and polymerization, although not evaluated, were not ruled out by the authors. In this regard, pioneering studies on conjugated polyunsaturated fatty acids, specifically β-eleostearic acid, already established that oxygen-containing polymers were main oxidation products [28]. Later, comparative oxidation studies on 9,12- and 10,12-methyl linoleate suggested that carbon-to-oxygen polymerization occurred rather than carbon-to-carbon polymerization [29] and that the peroxide decomposition is not a major factor in the mechanism of oxidation of conjugated substances [30]. Recently, based upon bond dissociation energy values, Oyman and coworkers [31] have suggested that the preferred reaction pathway for a free radical in tung oil, a drying oil containing substantial contents of the conjugated triene fatty acid α-eleostearic acid, is the addition to a conjugated double bond rather than abstracting a monoallylic hydrogen like in the propagation step of the oxidation of non-conjugated unsaturated fatty acids. By comparison with linseed oil, a drying oil containing substantial contents of the non-conjugated triene fatty acid α-linolenic acid, these authors observed lower absorption of oxygen and, in agreement with the results obtained for SO and TO in the present study, much lower PV in tung oil.

In order to examine the effectiveness of tocopherol homologues, alone and in combination with APL, the SO and TO stripped of their naturally occurring antioxidants were used and assays were carried out in Rancimat at 100 °C. OSI values for the antioxidant-stripped SO and TO were rather low, 2.2 and 2.8 h, respectively. Figure 4 represents the protection factors provided by the antioxidants tested.

In general, TO seemed less protected than SO by the antioxidants used, especially the samples containing tocopherol along with APL added. In the case of SO, α-tocopherol and α-tocopherol with APL were the least protected samples as compared with their counterparts, while δ-tocopherol and δ-tocopherol with APL were the most effective antioxidants. For the most stable samples, *i.e.*, those containing tocopherol along with APL, the order of antioxidant effectiveness was δ- > γ- > α-. These results are in agreement with other studies that show an order of antioxidant effectiveness in the opposite direction to the hydrogen-donating power, *i.e.*, δ- > γ- > α-, in fats and oils under different experimental conditions [32–35]. For the same substrate, concentration and oxidation conditions, the relative activities of the tocopherols are not only dependent on their chemical reactivity toward peroxyl and other free radicals, but also on many other possible side reactions that are likely to affect α-tocopherol to a greater extent [36]. As to the synergistic combination used, it has also been reported that the antioxidant effect of tocopherols greatly improves by addition of ascorbyl palmitate in polyunsaturated oils [37,38], being able to regenerate tocopherol.

As to the TO samples, any of the individual tocopherols studied provided a poor protection, which was similar for the three antioxidants. Unlike the SO, results for the combination of tocopherols with APL showed the lowest protection factor for δ-tocopherol and the highest for α- and γ-tocopherol, being similar for both.

Figure 4. Protection factors (ratio between the oil stability index in the presence and absence of antioxidants) provided by tocopherol homologues alone and in combination with APL in safflower oil (SO) and Tonalin oil (TO) stripped of their naturally occurring antioxidants in Rancimat at 100 °C. Data are expressed as Means ± Standard Deviations (n = 3). In each oil, different letters mean significant differences ($p < 0.05$). Toc: Tocopherol; APL: Ascorbyl Palmitate and Lecithin.



The effectiveness of tocopherol homologues, α-, β-, γ- or δ-tocopherol, on CLA has recently been studied at 50 °C by Ko and coworkers [17]. The results showed that δ- and γ-tocopherol exhibited the highest activity, whereas α-tocopherol had the lowest protective effect. Nevertheless, the results were obtained through analyses of PV and TBARS only. A misleading picture of the development of oxidation of CLA could have been obtained, given that hydroperoxides (PV) or thio-barbituric acid reactive substances, mainly malondialddehyde, are not the major oxidation compounds formed in CLA oxidation. To our knowledge, similar studies on CLA-rich oils have not yet been published.

4. Conclusions

The results obtained in this study reflect that different oxidation pathways could be involved in LA-rich and CLA-rich oils, the latter showing an oxidation pattern in which polymers are the main group of compounds formed. It appears that polymer quantitation can be an appropriate measurement to follow-up oxidation of CLA-rich oils *vs.* peroxide value, which is at present the only determination included in the quality specifications in relation to oil oxidation level. Also, the effectiveness of tocopherols combined with APL in TO, usually found in CLA-supplemented foods, has been found to be much lower than expected, thus demanding further research on antioxidant mode of action and efficiency in CLA-rich oils.

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Conflicts of Interest

The authors declare no conflict of interest.

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3.9.- La microencapsulación de aceites ricos en ácido linoleico conjugado (CLA) con componentes de leche desnatada protege frente a la polimerización.

Microencapsulation of conjugated linoleic acid (CLA)-rich oils with skimmed milk components protects against polymerization.

Francisca Holgado, María del Carmen García-Martínez, Joaquín Velasco, María Victoria Ruiz-Méndez y Gloria Márquez-Ruiz.

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Resumen:

Actualmente, los aceites ricos en ácido linoleico conjugado (CLA) están siendo utilizados en alimentos funcionales por los efectos beneficiosos para la salud que algunos isómeros CLA han demostrado. En este trabajo, la microencapsulación de un aceite rico en CLA (aprox. 80% de una mezcla de *9c, 11t* y *10t, 12c*- ácido linoleico) con componentes de leche desnatada se propone como método de protección frente a la oxidación y polimerización. Se evaluaron las principales características físico-químicas, como la temperatura de transición vítrea y el tamaño y la distribución medios de los glóbulos de aceite. Las muestras de CLA microencapsulado, intactas o tras eliminación de la fracción de aceite libre, se oxidaron a 30ºC en condiciones de oscuridad durante 3 meses. Durante su almacenamiento, se extrajeron y analizaron las fracciones de aceite libre y encapsulado mediante la determinación de la pérdida de sustrato (GLC-FID), determinación de polímeros (HPSEC-RID) y contenido en tocoferoles (HPLC-FD). Los resultados mostraron que el aceite libre se oxidó a mayor velocidad que el aceite encapsulado. En las fracciones encapsuladas, se acumularon elevadas cantidades de polímeros aun quedando considerables niveles de tocoferoles. En las muestras desprovistas de la fracción de aceite libre, se produjo un cambio drástico en las propiedades físicas, sin embargo, el aceite polimerizó en un grado similar al correspondiente a las fracciones encapsuladas de las muestras intactas.

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ORIGINAL ARTICLE

Microencapsulation of Conjugated Linoleic Acid (CLNA)-Rich Oil with Skimmed Milk Components Protects against Polymerization

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Abstract Oils rich in conjugated linoleic acid (CLNA) are currently used in functional foods because of the positive health effects of some CLNA isomers. In this work, microencapsulation of a CLNA-rich oil (containing approx. 80% of a mixture of *9c, 11 t,* and *10 t, 12c-* linoleic acid) with skimmed milk components is proposed as a means to protect it from oxidation and polymerization. The main physicochemical characteristics, i.e. glass transition temperature, mean oil globule size, and distribution, were evaluated. Microencapsulated samples, both intact and devoid of free oil fraction, along with the bulk oil, were oxidized at 30 °C in the dark for 3 months. Throughout storage, free and encapsulated oil fractions were separately extracted from samples and analyses including substrate loss by GLC-FID, polymer determination by HPSEC-RID, and tocopherol content determination by HPLC-FD were performed. Results showed that free oil oxidized much more rapidly than encapsulated oil. High amounts of polymers accumulated in samples with considerable high levels of tocopherols remain in encapsulated oil fractions. In samples devoid of free oil, a drastic change in the physical properties occurred but the oil polymerized to a similar extent to that found in the encapsulated fractions of intact samples.

Keywords Conjugated linoleic acid · CLNA · Oxidation · Microencapsulation · Polymerization

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Introduction

Conjugated linoleic acid (CLNA) is a collective term of a group of positional and geometric isomers of linoleic acid formed by rumen bacterial hydrogenation combined with mammalian delta 9-desaturation. The proportion of CLNA ranges from 0.12% to 0.68% of total fat in beef and from 0.34% to 1.07% of total fat in milk (Fritsche et al., 1999). The most biologically active isomers of CLNA are *cis*-9, *trans*-11-linoleic acid (rumenic acid), which is the most abundant isomer in nature, and *trans*-10, *cis*-12-linoleic acid (Pariza et al., 2000; Yang et al., 2015). Anticarcinogenic and antiatherogenic effects have been attributed to the *cis*-9, *trans*-11 isomer (Ha et al., 1990; Masso-Welch et al., 2004), while the *trans*-10, *cis*-12 isomer is claimed to promote weight loss and muscle-mass enhancement (Malpuech-Brugere et al., 2004; Whigham et al., 2007).

Nowadays, CLNA is used as a functional ingredient, especially in the form of oil (García-Martínez and Márquez-Ruiz, 2009). Commercial CLNA-rich oils are mostly obtained through alkali isomerization of safflower or sunflower oils (Saebo, 2003), and contain approximately 80% of CLNA with almost equal amounts of *9c, 11 t* and *10 t, and 12c-* linoleic acid, as well as trace amounts of other isomers. One of the most used CLNA-rich oil is Tonalin TG 80, constituted by mainly triacylglycerols (approx. 80%) and diacylglycerols (approx. 20%). As all polyunsaturated fatty acids, CLNA is highly susceptible to oxidation, and therefore, effective food supplementation with CLNA should guarantee protection of these bioactive fatty acids. Microencapsulation is a powerful strategy to prepare food ingredients that require protection

from oxidation and other chemical deterioration like loss of flavors or vitamins (Dias et al., 2015; Gharsalloui, Roudart, Chambin, Voilley & Saurel, 2007; Shahidi and Han, 1993), and especially oils rich in long-chain polyunsaturated fatty acids (Bakry et al., 2016; Drusch and Mannino, 2009; Márquez-Ruiz et al., 2003). Hence, it has also been proposed to enhance the oxidative stability of CLNA in a few studies (Choi et al., 2010; Costa et al., 2015; Jimenez et al., 2004, 2006; Kim et al., 2000; Lee et al., 2009).

In such studies, the acid form of CLNA was the only compound used and the only or main objective was to test different matrix components and analyze encapsulation efficiency and physicochemical properties. With the exception of Kim and coworkers (Kim et al., 2000), who used headspace-oxygen depletion to compare different cyclodextrins as encapsulating agents, and Jiménez and coworkers who compared losses of CLNA during storage at different relative water activities (Jimenez et al., 2004), only peroxide value (PV) was used as oxidation measurement and a range of very low values was generally reported. Besides and unfortunately, the free and the encapsulated fractions of microencapsulated CLNA throughout oxidation were not analyzed separately in neither of these studies, which is essential because of the heterogeneous oxidation rates observed in these complex, discontinuous systems (Morales et al., 2015; Velasco et al., 2006, 2009).

The mechanism of oxidation of conjugated systems is unclear and different pathways to those established for major, nonconjugated fatty acids seem to be involved (Brimberg and Kamal-Eldin, 2003; Yurawecz et al., 2003). In previous works, we demonstrated that PV or any other determination measuring hydroperoxides is not indicative of the oxidation state of CLNA substrates because polymer formation is otherwise the earliest and predominant event in the CLNA oxidation progress (García-Martínez et al., 2009; Luna et al., 2007; Márquez-Ruiz et al., 2014, 2016). Furthermore, we found that heptanal and *trans*-2-nonenal were volatile compounds exclusively formed from CLNA (García-Martínez et al., 2009; Márquez-Ruiz et al., 2016).

The objective of this work was to study the oxidative behavior of a CLNA-rich oil (Tonalin TG 80 oil) microencapsulated in skimmed milk components for the first time through separation of free and encapsulated oil fractions and using the determination of polymerization compounds as the most valid method to evaluate oxidation in CLNA substrates.

Materials and Methods

Materials

Tonalin TG 80 oil (TO) was acquired from Cognis Nutrition and Health (Cincinnati, OH, USA). Two batches of microencapsulated Tonalin oil samples (MT) were supplied by a local dairy manufacturer with the same preparation conditions that consisted in addition of TO to skimmed milk (1% w/v), two passes at 20 MPa for their homogenization (20,000 ± 1000 kPa), sterilization through the UHT indirect process (142 °C for 6 s), evaporation under vacuum (temperature below 72°C), and spray drying by atomization (air inlet temperature: 185°C, air outlet temperature: 90°C). The dried product contained theoretically 10% oil (minimum 6% TO), 36% proteins (29.5% caseins and 6.5% whey proteins), and 54% lactose. Tocopherol standards (α-, γ- and δ-) (purity >99%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals used were of analytical grade and obtained from local suppliers.

Oxidation Conditions

MT and TO samples were stored under nonlimited oxygen conditions in a desiccator containing silica gel, at 30 °C in the dark.

Lipid Extractions

Total Oil

The procedure applied was based on the Rose-Gottlieb method (Richardson, 1985), widely accepted for quantitative determination of fat in milk and milk powders. Briefly, 4 g of MT was dispersed in 40 mL of water heated at 65 °C in a 250 mL flask. A volume of 8 mL of 30% NH_4OH was added, the solution was gently stirred, the flask was closed, and heated at 65 °C for 15 min in a shaking water bath. Then, the solution was cooled at room temperature and the oil was extracted by applying three liquid–liquid extractions as follows: first, 20 mL ethanol, 50 mL diethyl ether, and 50 mL hexane; second, 10 mL ethanol, 50 mL diethyl ether, and 50 mL hexane; and third, 50 mL diethyl ether and 50 mL hexane. The components in each mixture of solvents were added separately and gentle shaking was applied after each addition. The organic phase was filtered through a filter paper containing anhydrous Na_2SO_4 and then the solvent was evaporated in a rotary evaporator at 40 °C. The extracted oil was finally dried to constant weight using a stream of nitrogen.

Free Oil

The free oil fraction was extracted according to Sankarikutty et al. (1988). A volume of 200 mL of n-hexane was added to 10 g of MT. Then, stirring was applied for 15 min at room temperature. After filtration through a filter paper, the solvent was evaporated in a rotary evaporator and the

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extracted oil was dried to constant weight by using a stream of nitrogen.

Encapsulated Oil

Partial extraction of encapsulated oil consisted of disruption of the solid matrix with a mortar and pestle and subsequent extraction of the lipids released with hexane. A 3 mL volume of water was added to 10 g of MT and the mixture was rubbed with a mortar and pestle until a dough was made. Then, 5 g of anhydrous Na_2SO_4 were added and mixed with the mortar and pestle to retain the water. The lipids were obtained by three extractions with 70 mL of hexane. After filtration through a filter paper, the solvent was evaporated at 40 °C in a rotary evaporator. The remaining solvent was removed from the extracted lipids with a stream of nitrogen at room temperature (Velasco et al., 2006).

Physicochemical Properties

Microencapsulation Efficiency (ME)

ME was determined from the quantitative extraction procedures described earlier for the total and the free oil fractions as follows:

$$\mathrm{ME}\,(\%) = \frac{(\text{Total oil} - \text{Free oil})}{\text{Total oil}} \times 100.$$

Oil Droplet Size

Analysis of the oil droplet size was performed in the powders reconstituted in water at a weight ratio of 1:7. The emulsions were readily reconstituted by dispersing the dried samples in deionized water at room temperature and applying vigorous shaking. A Malvern Mastersizer X (Malvern Instruments, Malvern, UK) operating with a 2 mW He—Ne laser beam (λ = 633 nm) and a 45 mm lens (size range 0.05–80 μm) was used. A relative refractive index η_{oil}/η_{water} = 1.095 and an absorption value of 0.1 were used in the calculations (Holgado et al., 2013).

Water Activity

The water activity of the microencapsulated samples was measured using a PawKit hygrometer (Decagon Devices Inc., Pullman, WA, USA).

Glass Transition Temperature (Tg)

The Tg was determined using a DSC Q 2000 calorimeter (TA Instruments, New Castle, DE, USA). Calibration of heat flow and temperature was performed with indium as standard (mp = 156.6 °C, ΔH_{fus} = 28.5 J g^{-1}). An aliquot of 10 mg MT was hermetically sealed into a 40 μL aluminum DSC crucible. An empty sealed aluminum crucible was used as reference. Heating runs at a rate of 10 °C min^{-1} were used from 0 to 125 °C. Duplicates and rescans were performed in each case to make sure that endothermic changes of the baseline corresponded to the Tg. The Tg was determined by the automatic mode of Universal Analysis 2000 (TA instruments), that is, the inflection point of the endothermic baseline shift.

Color Measurements

Color data were measured in the CIE 1976 (L*, a*, b*) color space using a CM-3500d Konica Minolta colorimeter in a 400–780 nm range.

Time for Powder Reconstitution in Water and pH

The time for powder reconstitution in water was measured at 26 °C using 2 g of the sample and 50 mL deionized water. Stirring at 300 rpm was applied and the time required for a complete dissolution was determined (Jimenez et al., 2010). Afterward, the pH of the corresponding reconstituted emulsions was measured.

Fatty-Acid Composition

Fatty-acid composition was determined in TO and in the extracted oil fractions from MT samples. Previous derivatization to fatty-acid methyl ester (FAME) with 2 M KOH in methanol, FAME were analyzed by GC- 6850 (Agilent Technologies, Palo Alto, CA, USA) equipped with a FID detector. FAME (c = 50 mg mL^{-1} in hexane, volume injected = 2 μL) were separated using a HP Innowax capillary column (30 m × 0.25 mm id, 0.25 μm film thickness). The temperature program used was 180 °C for 2 min, followed by 3 °C min^{-1} increase to 230 °C, and held there for 20 min. The temperatures of the injector and detector were held at 250 °C. Hydrogen was the carrier gas at a flow rate of 1 mL min^{-1} with a split ratio of 1:40 (IUPAC, 1992a, 1992b).

Determination of Dimers and Polymers

Polymerization compounds were determined following the IUPAC standard method (IUPAC, 1992c). TO and extracted oil fractions from MT were dissolved in THF (50 mg mL^{-1}) and analyzed in an HPSEC chromatograph equipped with a Rheodyne 7725i injector with a 10 μL sample loop, a Knauer 1200 HPLC pump (Knauer, Germany), and a Waters 2414 refractive index detector

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(Waters, Milford, MA, USA). The separation was performed on two 100- and 500-Å Ultrastyragel columns (25 cm × 0.77 cm i.d., 10 μm film thickness) packed with porous, highly cross-linked styrene-divinylbenzene copolymers (Agilent Technologies, Palo Alto, CA, USA) connected in series, with tetrahydrofuran (1 mL min^{-1}) as the mobile phase. The peaks resolved by HPSEC corresponding to polymerization compounds were triacylglycerol polymers (P) and triacylglycerol dimers (D).

Determination of Tocopherols

Analysis of tocopherols was performed by normal-phase HPLC with fluorescence detection according to the IUPAC Standard Method (IUPAC, 1992d).

Determination of Peroxide Value

PV was determined by the standard iodometric method, according to the (AOCS, 1998). Samples of 100 mg of oil and a 0.01 mol L^{-1} $Na_2S_2O_3$ solution were used.

Determination of the Oxidative Stability Index (OSI)

OSI was determined using a 743 Rancimat device (Metrohm, Switzerland). Samples of bulk oil (2.5 g ± 0.1 g) were placed into Rancimat standard tubes and subjected to the normal operation of the test by heating at 100 °C with a flow of air of 20 L h^{-1}. Evaluation mode 1 provided by the test as the intersection point of the two extrapolated straight parts of the conductivity curve was taken as the OSI.

Statistical Analysis

Characterization data were obtained by using three determinations. The oxidation experiments were carried out in triplicate and the results for polymerization compounds and tocopherols were expressed as mean values. Comparisons between means were made by applying one-way ANOVA using SPSS Statistics version 17.0 (SPSS Inc., Ireland). Differences between means were determined using Tukey's test. Significant differences were established at $P < 0.05$.

Results and Discussion

Characterization of Oil and Microencapsulated Oils

Table 1 shows fatty-acid and tocopherol compositions, and oxidative parameters of the TO used to prepare the MT samples. As can be observed, the contents of main isomers of CLNA, 9*c*, 11 *t* and 10 *t*, and 12*c* were very similar and sum up almost 80%. The level of total tocopherols was 732 mg kg^{-1}, γ- tocopherol being the most abundant. The PV and initial polymer content were low and typical of fresh refined oils.

Table 1 Characterization of Tonalin oil (TO)

	TO
Fatty-acid composition (wt.%)	
16:0	2.4 ± 0.0
18:0	2.6 ± 0.1
18:1	14.2 ± 0.2
18:2 (*9c, 12c*)	
18:2 (*9c, 11 t*)	39.2 ± 0.5
18:2 (*10 t, 12c*)	38.6 ± 0.4
Others	3.0 ± 0.0
Tocopherol composition (mg kg^{-1})	
A	81 ± 4
Γ	416 ± 10
Δ	235 ± 8
Peroxide value (meq O_2 kg^{-1})	2.5 ± 0.2
Dimers + Polymers (wt.%)	0.6 ± 0.1
Oxidative stability at 100 °C (h)	5.74 ± 0.0

TO, Tonalin oil.
Results are expressed as mean ± standard deviation ($n = 3$).

Table 2 shows main physicochemical characteristics and Table 3 shows fatty-acid and tocopherol compositions, and oxidative parameters, of initial samples of MT1 and MT2 batches and MT devoid of free oil fraction (MT2b). Fig. 1 shows the oil size distribution profiles and DSC thermograms of MT1, MT2, and MT2b. The oil content was similar in both batches (about 8%) and high encapsulation efficiencies were obtained, although significantly higher for MT2. Oil droplet size characteristics were similar for all samples. Values of Tg for MT1 and MT2 were, as expected, close to that of the carbohydrate contained in the skimmed milk, i.e., lactose (Thomas et al., 2004). However, after removal of the free oil fraction of MT2, it was noted that a drastic drop of Tg occurred and oil globule dispersion decreased, attributable to the structural changes in the matrix during washing with hexane and redrying. However, oil globule size parameters and a_w were similar for MT2 and MT2b, and solubility was only slightly slower in MT2b, even though some authors believe that the opposite is likely to occur because oil in the surface of particles may partly impede the contact between the matrix and water (McNamee et al., 1998).

Fatty-acid composition of MT1, MT2, and MT2b (Table 3) showed marked differences with respect to TO (Table 1), specifically the CLNA isomer proportion was significantly lower in microencapsulated oil samples while, conversely, contents of 16:0 and 18:0 were significantly

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Table 2 Physicochemical properties of microencapsulated TO samples (MT 1, MT 2, and MT 2b)

	MT 1	MT 2	MT 2b
Lipid distribution			
Total oil (g per 100 g TM)	8.12 ± 0.50	8.15 ± 0.45	
Free oil (g per 100 g TM)	0.97 ± 0.03a	0.51 ± 0.02b	
Encapsulation efficiency (%)	88.0 ± 1.62a	93.7 ± 1.30b	
Oil droplet size (lens 45 mm)			
$d_{(v,\ 0.5)}$ (μm)	0.62 ± 0.01a	0.74 ± 0.02b	0.70 ± 0.05b
$d_{(v,\ 0.9)} - d_{(v,\ 0.1)}$ (μm)	10.52 ± 1.26a	9.31 ± 0.87a	7.52 ± 0.24b
D[3,2] (μm)	0.60 ± 0.04	0.58 ± 0.05	0.55 ± 0.01
SA (μm^{-1})	10.00 ± 0.14	10.34 ± 0.30	10.91 ± 0.15
Water activity, a_w	0.20 ± 0.00	0.18 ± 0.00	0.20 ± 0.00
Glass transition temperature, Tg (°C)	63.9 ± 0.5a	75.4 ± 0.6b	32.3 ± 0.8b
Color parameters			
L*(D65)	94.06 ± 0.14	94.39 ± 0.11	94.41 ± 0.07
a*(D65)	−1.73 ± 0.04a	−1.72 ± 0.04a	−2.15 ± 0.17b
b*(D65)	11.99 ± 0.14a	13.32 ± 0.15b	12.60 ± 0.14b
Solubility, *t* (s)	260 ± 1a	230 ± 1b	245 ± 1b
pH	6.7 ± 0.04	6.7 ± 0.05	6.53 ± 0.05

MT, microencapsulated Tonalin oil; SA, surface area; TO, Tonalin oil.

Results are expressed as mean ± standard deviation ($n = 3$).

Different letters in the same row indicate significant differences ($P < 0.05$).

Table 3 Characterization of extracted oils from initial microencapsulated TO samples (MT 1, MT 2, and MT 2b)

Fatty-acid composition (%)		MT 1		MT 2		MT 2b
		Free	Encapsulated	Free	Encapsulated	
16:0		6.8 ± 0.2	6.6 ± 0.2	3.7 ± 0.3	3.0 ± 0.2	3.1 ± 0.1
18:0		5.2 ± 0.2	4.1 ± 0.0	3.3 ± 0.2	2.9 ± 0.0	3.0 ± 0.1
18:1		14.2 ± 0.0	15.2 ± 0.1	13.9 ± 0.0	14.4 ± 0.1	14.5 ± 0.2
18:2	(9*c*,11*t*)	33.8 ± 0.6	34.2 ± 0.7	38.4 ± 0.6	39.0 ± 0.7	38.7 ± 0.5
	(10*t*,12*c*)	34.1 ± 0.4	34.2 ± 0.5	37.1 ± 0.4	37.7 ± 0.5	37.0 ± 0.4
Others		5.9 ± 0.3	5.8 ± 0.4	3.6 ± 0.3	3.1 ± 0.2	3.3 ± 0.3
Tocopherols (mg kg^{-1})		502 ± 15a	691 ± 17b	683 ± 20	720 ± 18	652 ± 15
Dimers + Polymers (%)		0.5 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1

MT, microencapsulated Tonalin oil; TO, Tonalin oil.

Results are expressed as mean ± standard deviation ($n = 3$).

Different letters in the same row indicate significant differences between values of free and encapsulated fractions ($P < 0.05$).

higher. Also, short- and medium-chain fatty acids were detected in MT samples. This is attributable to the residual milk fat present in skimmed milk, as already reported (Rodríguez-Alcalá and Fontecha, 2007). Also, it was clear that MT1 samples contained more milk fat, as reflected in slightly higher contents of 16:0 and 18:0 and lower contents of CLNA isomers. Total tocopherol contents were lower in general in microencapsulated oil samples than in TO and decreased levels were detected in the free compared to the encapsulated oil fractions in both MT1 and MT2. Moreover, MT2b presented even lower tocopherol content than the encapsulated oil of MT2, and this loss was probably due to the further sample manipulation, i.e. washing with a solvent and redrying.

Polymerization in Microencapsulated Oils

Fig. 2 shows the results obtained throughout storage of all samples at 30 °C, including bulk TO. The results of MT1 and MT2 showed similarly that free oil oxidized much more rapidly than encapsulated oil, as occurred in the case of TO. In free oil fractions of MT samples and TO, polymerization began very soon while in encapsulated fractions only after 40–50 days significant levels of dimers and

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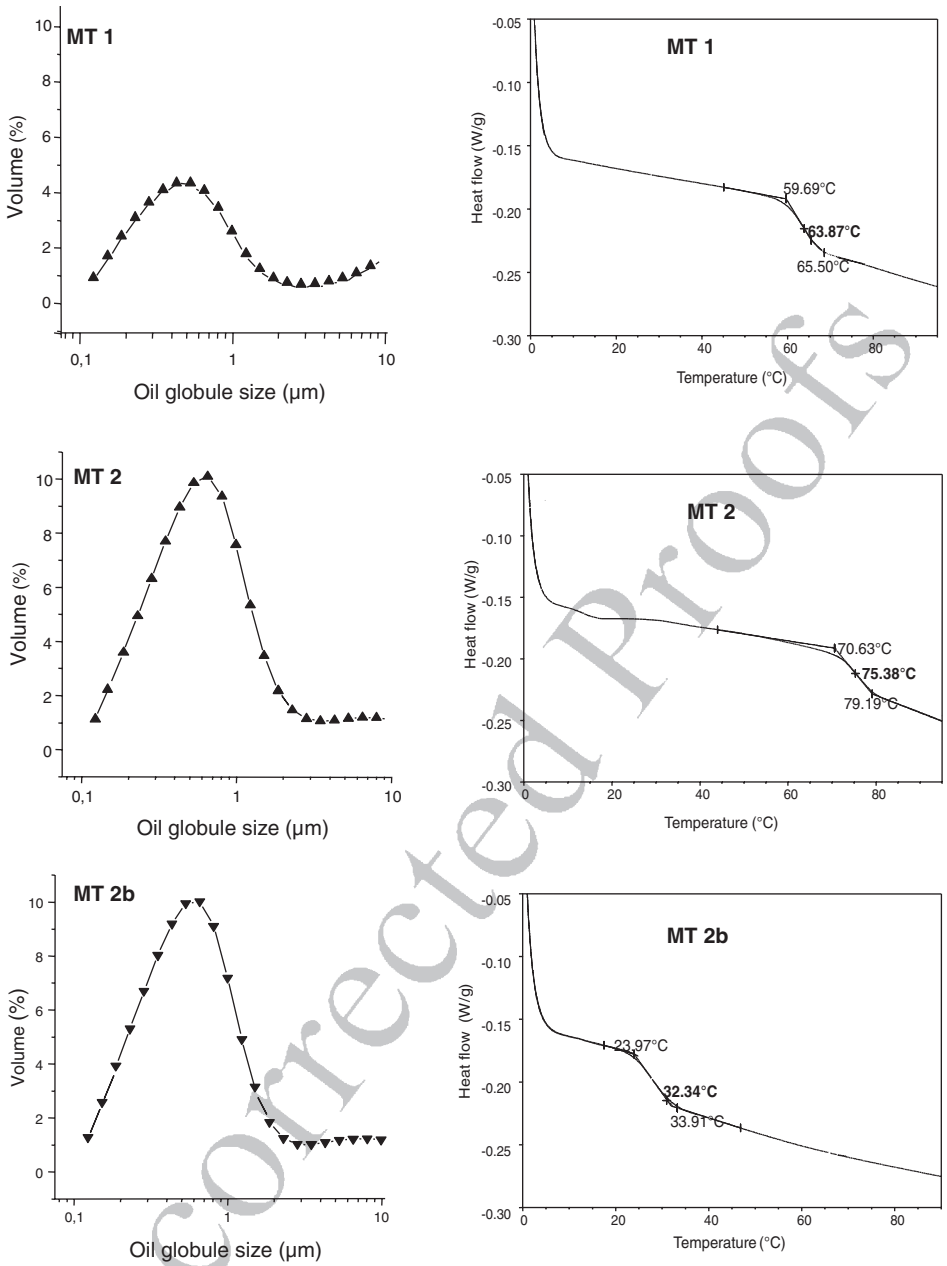

Fig. 1 Oil globule size distribution and differential scanning calorimetry thermograms of microencapsulated Tonalin oil samples (MT1 and MT2) and microencapsulated Tonalin oil devoid of free oil fraction (MT2b)

polymers were formed. Therefore, the protection exerted by the skimmed-milk-component matrix against oxidation of the oil embedded or encapsulated was relevant. Dairy-like matrices have been previously reported to effectively protect lipids prone to oxidation such as fish oils (Keogh et al., 2001; Velasco et al., 2000, 2006), and caseins seem to play an essential role in such a protective effect (Drusch et al., 2007). In the present work, results showed that, when the antioxidants were exhausted, contents of dimers and polymers were already high but quite different between the free oil fractions (about 5%) and the encapsulated fractions (about 8–10%). In other words, in encapsulated oil fractions, high amounts of polymers accumulated in samples with considerably high levels of tocopherols remaining.

The only difference observed in the oxidative patterns of MT1 and MT2 was the oxidation rate of the encapsulated oil fraction, higher for the former. Thus, MT 1 was totally depleted of tocopherols and had 10% dimers and polymers after 80 days of storage while MT2 at 77 days kept half the tocopherol content and showed only 2.2% dimers plus polymers. Differences between oxidative stability of MT1 and MT2 were not attributable to any of the physicochemical

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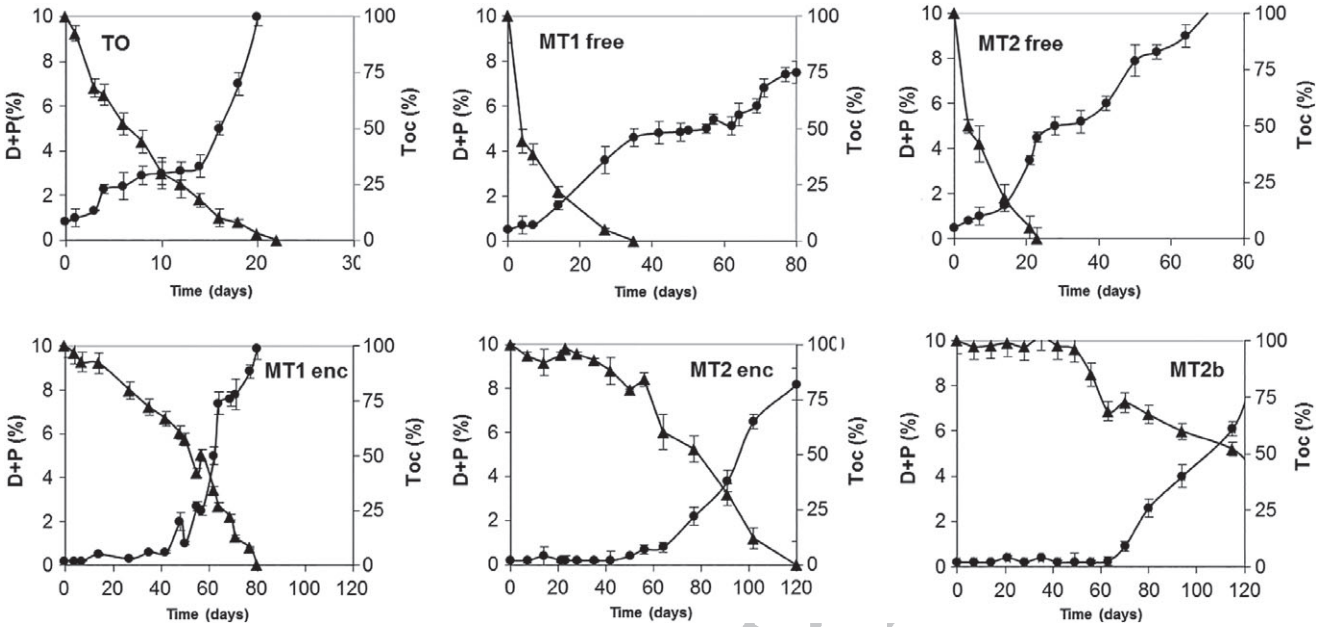

Fig. 2 Time course of formation of dimers and polymers (D + P) (circle) and retention of tocopherols (triangle) in bulk Tonalin oil samples (TO), free and encapsulated (enc) oil fractions of microencapsulated Tonalin oil samples (MT1 and MT2) and in microencapsulated Tonalin oil devoid of free oil fraction (MT2b) during oxidation at 30°C in the dark. Values correspond to means of triplicate samples and error bars to standard deviations

properties or oxidative parameters analyzed in the starting samples (Tables 2 and 3), and reflected the influence of other variables involved in the preparation process.

When samples were devoid of free oil (MT2b), polymerization occurred to a similar extent to that found in the encapsulated fractions of intact samples. However, loss of tocopherols in MT2b was comparatively retarded from 65 days of storage, which may be attributed to increased protection of the encapsulated oil fraction following washing with hexane and redrying because of structural changes that resulted in lower oxygen permeability.

From the data shown in Fig. 2, the estimated rate constants for tocopherol loss obtained were much higher in free oil fractions (10.4×10^4 days^{-1} and 13.6×10^4 days^{-1} for MT1 and MT2, respectively) than in encapsulated oil fractions (0.25×10^4 days^{-1} and 0.16×10^4 days^{-1} for MT1 and MT2, respectively). Furthermore and most relevant were the differences found in the ratio of remnant tocopherols-to-polymerization compounds between free and encapsulated oil fractions of MT1 and MT2, as illustrated in Fig. 3. Clearly, the proportion of polymerization compounds formed in relation with tocopherol levels during the induction period was much higher in encapsulated than in free oil fractions. These results are attributed to the discontinuous oxidation of encapsulated oil, i.e., the coexistence of oil globules at different oxidation extents (from oil globules slightly oxidized with high contents of antioxidants to others highly polymerized and lacking of antioxidants) (Morales et al., 2015; Velasco et al., 2006, 2009).

To our knowledge, only five studies have been published on oxidation of microencapsulated CLNA (Costa et al., 2015; Jimenez et al., 2004, 2006; Kim et al., 2000; Lee et al., 2009). In all these studies, as commented in the Introduction, the acid form of CLNA was used and separate analysis of the free and the encapsulated fraction of microencapsulated CLNA throughout oxidation was not carried out. Analysis of oxidation was normally approached by PV measurement and substrate loss determination.

As to PV, the results obtained in our lab on CLNA model systems and CLNA-rich oils have demonstrated that polymer formation and not hydroperoxide formation occur in CLNA systems from the beginning of the oxidation process (García-Martínez et al., 2009; Luna et al., 2007; Márquez-Ruiz et al., 2014, 2016). This finding was opposite to what was expected and found in nonconjugated systems thus invalidating PV to control oxidation in CLNA systems. In fact, PV obtained by Jiménez and coworkers in microencapsulated CLNA during storage were very low (Jimenez et al., 2004, 2006) and even though authors did not discuss these results, they clearly indicated that hydroperoxide formation was minor and not representative of the oxidation state. For example, they reported that samples of CLNA microencapsulated in whey proteins stored at 35 and 45°C showed CLNA losses above 50% and PV lower than 5 meq O_2 kg^{-1} (Jimenez et al., 2004). In a previous work on CLNA oils, we proposed a pathway that can account for the low hydroperoxide amounts formed during oxidation of CLNA substrates, which is the preferential

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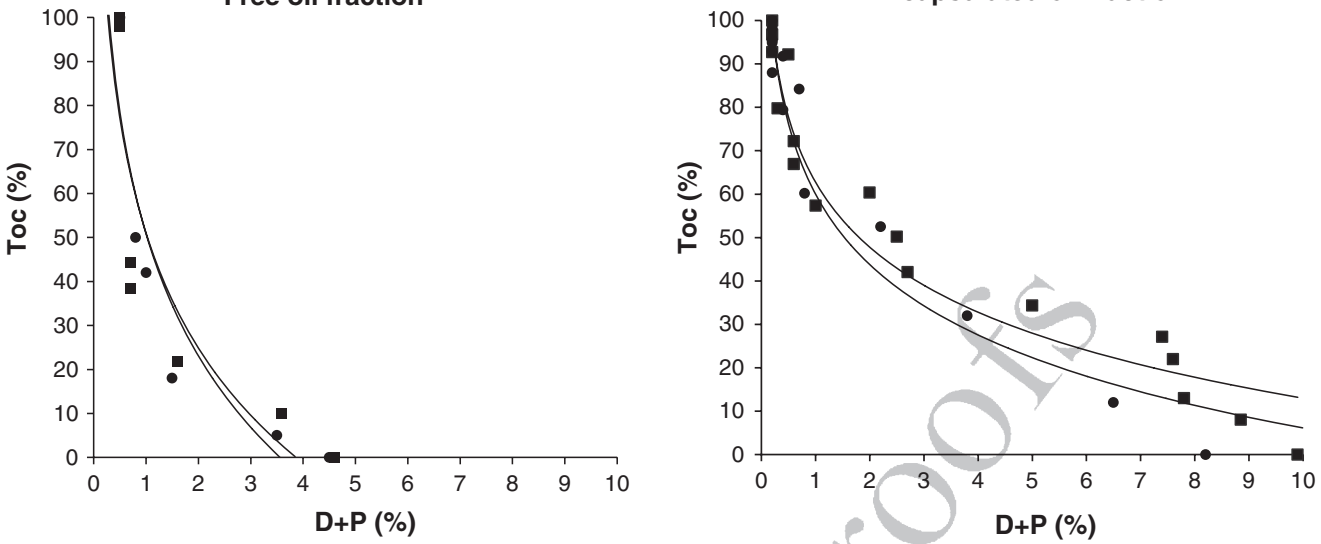

Fig. 3 Ratios of tocopherols (Toc)-to-dimers (D) and polymers (P) in free and encapsulated oil fractions of microencapsulated Tonalin oil samples MT1 (square) and MT2 (circle) oxidized at 30°C in the dark

addition of the peroxyl radicals formed during the propagation step to the conjugated diene systems thus leading to peroxyl radical dimers ultimately yielding polymeric peroxides (Márquez-Ruiz et al., 2016). Therefore, such polymers cannot be considered termination products characteristic of advanced oxidation stages but primary oxidation compounds formed during the propagation state. The results obtained in the present work confirm that polymers are formed in early stages of oxidation, before exhaustion of antioxidants, both in free oil and encapsulated oil fractions (Fig. 2).

With respect to substrate loss determination by gas–liquid chromatography, it is important to state that it is an indirect measurement because eluting nonoxidized FAME are quantified with addition of internal standard, and oxidized compounds are determined by difference. It is a measurement widely used to evaluate oxidation in general and has also been applied to microencapsulated CLNA (Costa et al., 2015; Jimenez et al., 2004). With the purpose of evaluating the utility of substrate loss determination in the present study, selected samples of MT1 encapsulated oil fractions were analyzed. Results are shown in Table 4. While formation of polymerization compounds was consistent from the beginning of oxidation and increased significantly at 48 days (2%), substrate loss measurement showed important fluctuations and only changed significantly after 64 days, when polymerization compounds were already 13.3%. Furthermore, sample replicates showed relative standard deviations much higher for substrate loss determination than for polymer quantitation. Costa and coworkers have also reported very high standard deviations in oxidative stability studies of CLNA microencapsulated in pea protein using substrate loss determination (Costa et al., 2015). These results are also in agreement with those previously obtained in our lab with model compounds of FAME, which showed the low sensitivity of the substrate loss determination and the high variability of the values obtained (Márquez-Ruiz et al., 2007).

Table 4 Evolution of substrate loss and formation of polymerization compounds in the encapsulated oil fraction of MT1 samples

Days	Substrate loss (%)		Dimers + Polymers (%)	
	Mean	RSD (%)	Mean	RSD (%)
0	0.0	0.0	0.2	8.3
4	1.1	62.0	0.2	9.4
7	0.8	54.2	0.2	9.1
14	3.2	47.2	0.5	8.5
27	1.2	205.4	0.3	9.9
35	0.4	32.1	0.6	6.2
42	0.6	148.5	0.6	8.0
48	4.3	33.5	2.0	18.7
50	4.7	22.0	1.0	6.5
55	3.8	29.5	2.7	7.0
57	1.8	165.4	2.5	8.8
62	3.9	59.3	5.0	7.2
64	13.3	32.2	7.4	8.3
69	13.5	10.4	7.6	4.2
71	13.9	11.2	7.8	5.9
77	13.7	9.8	8.9	3.9
80	29.3	5.6	9.9	5.1

MT, Microencapsulated Tonalin oil.

Results are expressed as mean ± relative standard deviation ($n = 3$ samples).

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Conclusions

Polymerization in CLNA-rich oil microencapsulated in skimmed milk components occurred since the beginning of the oxidation process and was markedly earlier and greater in the free oil fraction, hence showing the high protection provided by the encapsulation matrix. Oxidation of the encapsulated oil seemed to be unaffected by the removal of the free oil and subsequent drop of Tg thereby obtaining a functional ingredient of high oxidative stability. Also, results indicated the relevance of polymer formation and determination in microencapsulated CLNA-rich oils and clearly showed the heterogeneity of oxidation in the encapsulated oil fraction. Further research is needed to gain insights into the oxidation events in microencapsulated CLNA to guarantee protection of these bioactive but highly polymerizable fatty acids and preserve physical and chemical stability of functional foods containing them.

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Conflict of interest The authors declare they have no conflict of interest.

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4.- Discusión general.

El objetivo general de esta tesis fue abordar el estudio del proceso de oxidación en productos lácteos y los principales resultados obtenidos se discuten a continuación atendiendo a los objetivos específicos fijados:

4.1.- Determinación de los compuestos de oxidación en productos lácteos comercializados, tradicionales y funcionales.

Los resultados discutidos en este apartado y los antecedentes encontrados forman parte de las publicaciones:

- García-Martínez MC and Márquez-Ruiz G. Lipid oxidation in functional dairy products (2009) *Current Nutrition and Food Science* 5:209-216.
- García-Martínez MC, Fontecha J, Velasco J, Holgado F and Márquez-Ruiz G. Occurrence of lipid oxidation compounds in commercialized functional dairy products. (2018) *International Dairy Journal* 86:27-35.

Para determinar cuantitativamente los compuestos de oxidación en productos lácteos, fue necesario el desarrollo y aplicación de métodos específicos de extracción de lípidos en una gran variedad de productos lácteos así como de métodos cuantitativos que permitan una medida global y objetiva del proceso oxidativo desde los primeros estadíos hasta fases de oxidación avanzada. Se seleccionaron productos representativos de productos lácteos comercializados, tradicionales y funcionales: leche esterilizada y tratada térmicamente mediante el procedimiento UHT (UltraHighTemperature), leche en polvo, leche concentrada, yogures y fórmulas infantiles; así como preparados lácteos funcionales con ácidos grasos poliinsaturados (AGPI) omega-3, ácido linoleico conjugado (CLA) y estanoles vegetales.

La mayoría de los estudios realizados anteriormente se han basado en métodos que detectan compuestos formados durante una etapa concreta del proceso de oxidación, por ejemplo productos primarios, mediante el índice de peróxidos, o secundarios, mediante el test del ácido tiobarbitúrico o análisis de compuestos volátiles (García-Martínez y Márquez-Ruiz, 2009; Barriuso y col., 2013).

En primer lugar, se puso a punto una metodología basada en una combinación de técnicas cromatográficas de adsorción y exclusión para la evaluación de la oxidación lipídica en los diferentes productos lácteos seleccionados (Márquez-Ruiz y Dobarganes, 2006). Las muestras fueron evaluadas en el tiempo de adquisición y al final de su vida útil.

Los productos lácteos convencionales iniciales mostraron niveles ínfimos de oxidación, aunque se observó una ligera pero significativa mayor oxidación de la fracción de grasa superficial en la leche en polvo de productos lácteos convencionales. Los productos funcionales se caracterizaron por un elevado contenido de tocoferoles y mostraron sin embargo niveles más altos de oxidación inicial como era de esperar por su contenido en aceites poliinsaturados refinados, pero los valores fueron muy bajos y en el caso del índice de peróxidos, inferiores a 10 meq O_2/kg (límite establecido para aceites refinados). En los productos con CLA, resultó sorprendente detectar la presencia de polímeros de triacilgliceroles, ya que estos compuestos sólo se forman en estadíos muy avanzados de oxidación en los cuales ya están presentes cantidades significativamente altas de triglicéridos oxidados y dímeros, que sin embargo fueron muy bajos en estas muestras.

En cuanto a la evaluación de los productos en su fecha de caducidad, los resultados obtenidos demostraron que sólo determinados productos lácteos funcionales enriquecidos con AGPI omega-3 ó CLA fueron susceptibles a oxidación, ya que presentaron niveles significativamente más altos de triglicéridos oxidados o poliméricos. La Figura 1 recoge sólo los principales resultados obtenidos en muestras de productos lácteos funcionales ya que en las muestras de productos lácteos convencionales no se observó ningún cambio oxidativo, ni siquiera en la leche en polvo donde la fracción de grasa superficial está más expuesta pero cuyo envase al vacío ejerció la protección adecuada.

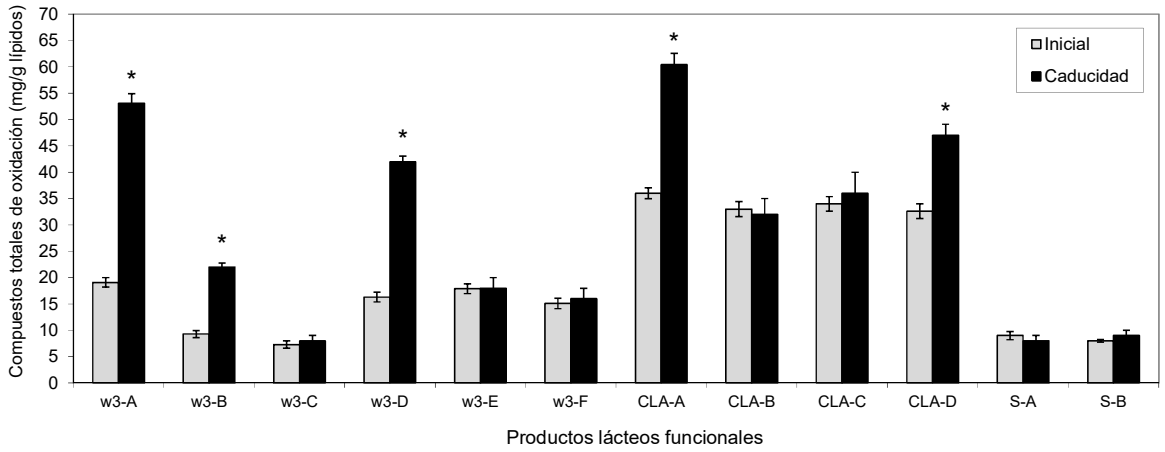

Fig. 1.- Cambios en compuestos totales de oxidación lipídica en productos lácteos funcionales (* indica diferencias significativas entre muestras iniciales y a fecha de caducidad). Abreviaturas; ω3, omega-3; CLA, ácido linoleico conjugado, S, estanol.

Como puede observarse, los productos lácteos con estanoles no experimentaron oxidación durante su periodo de vida útil, en concordancia con los resultados obtenidos por otros autores (Soupas y col., 2006; García-Llatas y col., 2013; González-Larena y col., 2015). En cuanto a los productos con AGPI omega-3, no se observó oxidación significativa en tres de ellos (ω–3-C, ω–3-E y ω–3-F) y es interesante comentar que en estos productos la adición del aceite de pescado se había realizado mediante un ingrediente microencapsulado. Está bien demostrado que la estabilidad de los productos lácteos disminuye cuando se enriquecen con aceites de pescado u otros aceites ricos en AGPI omega-3 (Let y col., 2003; 2004; 2015; Jacobsen, 2010; 2015; Saga y col., 2013). Sin embargo, la microencapsulación puede ejercer protección en estos ingredientes funcionales, además de enmascarar el olor y sabor típicos de los aceites de pescado (Beindorff y col., 2010).

La metodología aplicada, por primera vez en productos lácteos, permitió determinar los principales compuestos de oxidación mayoritarios. Como se muestra en la Tabla 1, en el caso de los productos con AGPI ω-3 el aumento de los niveles de oxidación se debe al incremento de triglicéridos monómeros oxidados mientras que en las muestras con CLA los compuestos poliméricos son los mayoritarios y sorprendentemente los valores de triglicéridos monómeros oxidados e índices de peróxidos permanecen bajos.

Tabla 1.- Parámetros oxidativos en productos lácteos funcionales a fecha de caducidad.

	ω3-A	ω3-B	ω3-D	CLA-A	CLA-D
IP (meq O_2/kg)	65,9 ± 4,16*	26,0 ± 3,01*	44,5 ± 5,01*	7.2 ± 5,18	8.3 ± 3,99
TG (mg/g):					
- OxTGM	41,1 ± 2,88*	19,1 ± 1,55*	34,5 ± 1,64*	8.0 ± 1,17	10.4 ± 1,88
- TGD	9,9 ± 1,05	3,2 ± 0,29	7,9 ± 0,87	3.5 ± 0,50	5.0 ± 1,01
- TGP	2,1 ± 0,14	ND	ND	48.9 ± 3,22*	32.8 ± 2,98*
TOC (mg/kg):					
– α	332,6 ± 11,41*	346,2 ± 13,10*	198,7 ± 9,31*	60.0 ± 2,91	21.8 ± 2,14
– β	29,1 ± 2,87	25,1 ± 1,04	ND	10.7 ± 0,53	ND
– γ	93,0 ± 7,18*	80,9 ± 4,59	20,0 ± 1,22	283.5 ± 10,18	309.9 ± 17,22
– δ	40,2 ± 3,04	36,1 ± 2,89	38,9 ± 3,03	201.4 ± 9,31*	154.2 ± 12,99*
Total	494,5 ± 14,11*	487,0 ± 14,22*	259,6 ± 9,87*	551.7 ± 14,11*	486.1 ± 21,68*

Datos expresados en Media ± DS, *n* = 3. Los asteriscos indican diferencias significativas entre muestras iniciales y a fecha de caducidad ($p < 0.05$). Abreviaturas: ω3, producto lácteo con ácidos grasos omega-3; CLA, producto lácteo con ácido linoleico conjugado; IP, índice de peróxidos; TG, triglicéridos; oxTGM, triglicéridos monómeros oxidados; TGD, dímeros de triglicéridos; TGP, polímeros de triglicéridos; TOC, tocoferoles; ND, no detectado.

La Figura 2 ilustra claramente las grandes diferencias observadas en las fracciones concentradas de compuestos oxidados analizadas por cromatografía de exclusión, correspondientes a las muestras ω-3-A y CLA-A a fecha de caducidad. Las muestras ω-3-A reflejan el proceso de autoxidación más ampliamente aceptado (Frankel, 2005), consistente en la formación inicial de hidroperóxidos, incluidos en el pico de los triglicéridos monómeros oxidados o alternativamente evaluados por el índice de peróxidos. En etapas subsiguientes de este proceso se forman compuestos monoméricos con funciones oxigenadas estables, como los grupos cetona, aldehído, epóxido o alcohol, también incluidos en el pico de los triglicéridos monómeros oxidados, y sólo en estadíos más avanzados tiene lugar la formación de dímeros y polímeros. Por tanto, la formación de polímeros de elevado peso molecular en las muestras con CLA unido a los sorprendentemente bajos niveles de índice de peróxidos encontrados indicó que la cinética de oxidación del aceite rico en CLA es diferente a la ampliamente aceptada para sustratos no conjugados y justificó la realización de estudios complementarios.

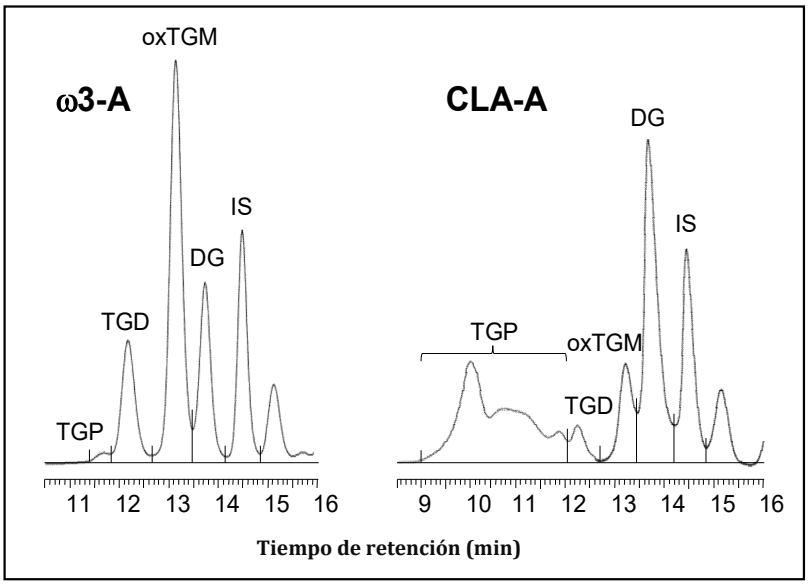

Fig. 2.- Cromatogramas de exclusión molecular de las fracciones polares de lípidos extraídos de los productos lácteos ω-3-A y CLA-A a fecha de caducidad. Abreviaturas: TGP, polímeros de triglicéridos; TGD, dímeros de triglicéridos; oxTGM, triglicéridos monómeros oxidados; DG, diglicéridos, IS, patrón interno (monoestearina).

En resumen, de todos los productos lácteos comercializados analizados, es importante destacar la susceptibilidad a la oxidación de los productos funcionales enriquecidos en ácidos grasos poliinsaturados omega-3 y en CLA. Concretamente, la oxidación de los lácteos enriquecidos en CLA hubiese pasado desapercibida de utilizarse exclusivamente el índice de peróxidos, que es la única determinación incluida en las especificaciones de calidad de estos productos. Sin embargo, la evaluación conjunta de todos los compuestos de oxidación no volátiles realizada en este estudio ha permitido detectar la formación de polímeros y permite proponer medidas de oxidación alternativas para el control de calidad de estos productos.

4.2.- Evaluación del efecto del tratamiento térmico de esterilización y de la adición de ácidos grasos poliinsaturados: Estudios de oxidación en fórmulas infantiles modelo.

Los resultados discutidos en este apartado forman parte de las siguientes publicaciones:

- García-Martínez MC, Holgado F, Velasco J and Márquez-Ruiz G. Effect of classic sterilization on lipid oxidation in model liquid milk-based infant and follow-on formulas (2012*) European Journal of Lipid Science and Technology* 114:1373-1380.
- Márquez-Ruiz G, García-Martínez MC and Holgado F. Effect of sterilization of bottled infant milk: impact on lipid oxidation and tocopherols, in "Handbook of dietary and nutritional aspects of bottle feeding", ed. VR Preedy, RR Watson and S Zibadi, Wageninen Academic Publishers, The Netherlands 2014, pp. 221-237.

Se han realizado estudios de oxidación específicos en productos lácteos muy susceptibles a la oxidación lipídica, las fórmulas infantiles, y de extraordinaria importancia porque suponen la única fuente de nutrientes de los lactantes y es esencial garantizar su calidad y seguridad. Las fórmulas infantiles constituyen un excelente modelo para determinar si el proceso de elaboración y el tratamiento de esterilización influyen en la oxidación lipídica ya que son particularmente susceptibles por su alto contenido en ácidos grasos poliinsaturados y minerales con acción prooxidante.

Se elaboraron fórmulas infantiles modelo que simularon fórmulas de inicio y continuación con una mezcla de aceites con la composición de ácidos grasos normalizada por la regulación española que recoge las especificaciones de la normativa europea (Real Decreto, 2008), y con fines comparativos dos fórmulas con uno de los aceites utilizados en la formulación, esto es, un aceite resistente a la oxidación (girasol alto oleico) y un aceite muy susceptible a la oxidación (pescado), con o sin hierro en la formulación. La relación caseinato sódico:proteínas del suero de la leche fue 1:4 and 4:1 para las fórmulas de inicio y las de continuación, respectivamente.

Los resultados obtenidos mostraron importantes pérdidas de tocoferoles durante el procesado, que incluyó el tratamiento de esterilización, y que el principal agente protector fue la fracción proteica, especialmente el caseinato sódico, presente en mayor concentración en las fórmulas de continuación. Estos resultados pueden atribuirse a la capacidad quelante de los grupos fosforilados de la caseína frente a los iones metálicos prooxidantes presentes en la emulsión (Elias y col., 2008).

En relación con el contenido en tocoferoles (Figura 3), como era de esperar, la mayor disminución respecto al aceite inicial se observó en las muestras preparadas con aceite de pescado. Los niveles de tocoferoles disminuyeron drásticamente (45-65%) en muestras exentas de proteínas y especialmente en las que contenían hierro. La pérdida superior de tocoferoles en las fórmulas de inicio respecto a las de continuación es atribuible al mayor efecto protector de las caseínas frente a las proteínas del suero de la leche. Otros estudios han demostrado igualmente que las pérdidas de tocoferoles en fórmulas infantiles pueden ser superiores al 50% durante su almacenamiento a temperatura ambiente (Hu y col., 2003; Miquel y col., 2004).

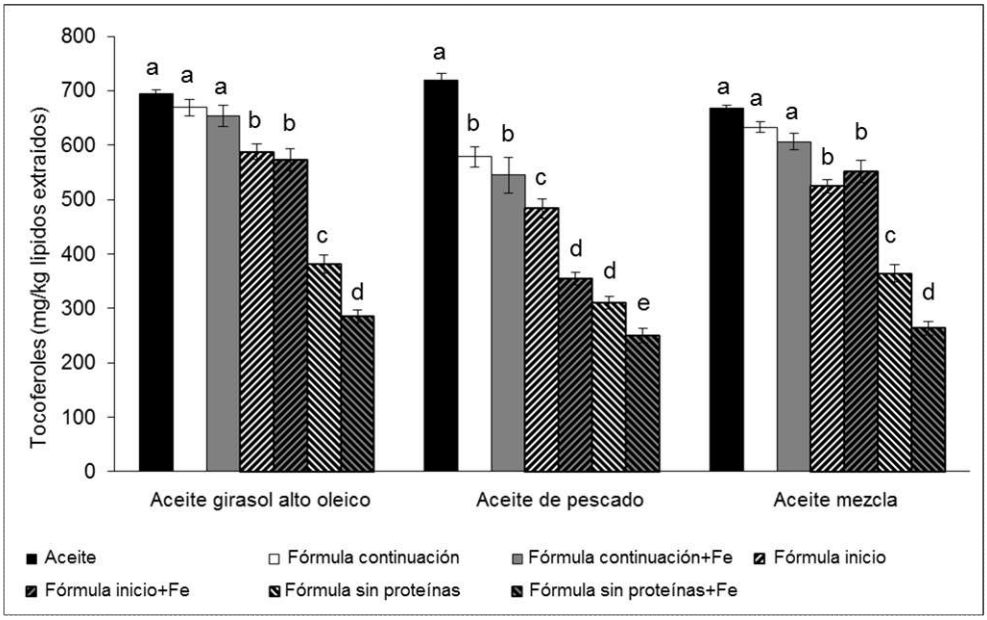

Fig. 3.- Niveles de tocoferoles en aceites iniciales y en los aceites extraídos de fórmulas infantiles modelo. Las letras diferentes indican diferencias significativas entre las muestras preparadas con el mismo aceite.

La Tabla 2 recoge sólo los resultados correspondientes a las fórmulas elaboradas con la mezcla de aceites, que reflejan claramente que las muestras más oxidadas son las exentas de proteínas y entre éstas que contenían hierro. Sin embargo, no se observaron diferencias en los parámetros de oxidación entre fórmulas iniciales y de continuación.

Tabla 2.- Niveles de oxidación en la mezcla de aceites utilizada en la preparación de fórmulas infantiles y en los aceites extraídos de las fórmulas infantiles

	Compuestos de oxidación (%)				Indice de peróxidos
	oxTGM	TGD	TGP	Total	[meq O_2/Kg]
Aceite mezcla	1,5	0,4	ND	1,9 ± 0.1 [a]	6,1 ± 0.1 [a]
Fórmula no proteínas	9,7	2,0	ND	11,7 ± 2.4 [b]	172,8 ± 18.1 [b]
Fórmula no proteínas+Fe	13,5	3,1	1,3	16,2 ± 3.7 [c]	260,1 ± 20.3 [c]
Fórmula inicio	1,7	0,8	ND	2,5 ± 0.4 [a]	9,5 ± 3.2 [a]
Fórmula inicio+Fe	1,6	0,6	ND	2,2 ± 0.3 [a]	9,2 ± 2.1 [a]
Fórmula continuación	1,3	0,7	ND	2,0 ± 0.3 [a]	7,1 ± 2.6 [a]
Fórmula continuación+Fe	1,5	0,6	ND	2,1 ± 0.4 [a]	6,2 ± 2.8 [a]

Datos expresados en Media ± DS, *n* = 3. Las letras diferentes indican diferencias significativas ($p < 0.05$). Abreviaturas: oxTGM, triglicéridos monómeros oxidados; TGD, dímeros de triglicéridos; TGP, polímeros de triglicéridos; Fe, hierro; ND, no detectado.

En resumen, el tratamiento de esterilización no indujo cambios oxidativos en fórmulas de inicio y continuación elaboradas con composición similar a las comercializadas aunque se observaron disminuciones significativas de tocoferoles en las fórmulas de inicio con la consecuente pérdida de protección frente a la posibles deterioros oxidativos durante el posterior periodo de comercialización.

4.3.- Evaluación del efecto de las condiciones de almacenamiento en la formación de compuestos oxidados en fórmulas infantiles comercializadas.

Los resultados discutidos en este apartado están incluidos en parte en la siguiente publicación:

- García-Martínez MC, Rodríguez-Alcalá LM, Marmesat S, Alonso L, Fontecha J and Márquez-Ruiz G. Lipid stability in powdered infant formula stored at ambient temperatures (2010) *International Journal of Food Science and Technology* 45:2337-2344.

En estos estudios se evaluó la influencia de las condiciones de conservación en la estabilidad oxidativa de fórmulas infantiles en polvo comercializadas y se introdujo por primera vez la determinación de dos fracciones diferenciadas de lípidos en fórmulas infantiles: la fracción superficial, obtenida mediante extracción con éter de petróleo; y la fracción encapsulada, obtenida tras disgregar la matriz de proteínas e hidratos de carbono en medio básico fuerte (con solución etanólica de amoníaco) y posterior extracción con éter dietílico y pentano. Las muestras fueron almacenadas a 25, 30 y 37ºC en cámaras termostatizadas durante 3 meses. Estos ensayos se realizaron en condiciones moderadamente aceleradas ya que las muestras se almacenaron en bolsas de plástico que aunque cerradas no estaban en atmósfera inerte o vacío.

Por otra parte, se almacenaron muestras en sus envases originales (latas) a 25ºC hasta la fecha de caducidad establecida por el fabricante (30 meses). Los productos habían sido envasados en atmósfera modificada constituida por nitrógeno (aproximadamente 80%) y CO_2, pero contenían cantidades residuales de oxígeno (3-5%).

Se realizó una completa caracterización físico-química de los productos, cuyos resultados constan en la Tabla 3, necesaria para evaluar la distribución de los lípidos en estos sistemas heterofásicos en polvo. Entre estos parámetros, es especialmente importante a considerar previo a la realización de ensayos de oxidación en productos en polvo, la temperatura de transición vítrea ya que los

ensayos siempre deben realizarse a temperaturas inferiores para evitar cambios estructurales en los productos.

Tabla 3.- Características físicoquímicas de las fórmulas infantiles.

Distribución lipídica:	
Aceite Total (g/100 g)	24,37 ± 1,77
Aceite Libre (g/100 g)	1,95± 0,08
Eficacia de Encapsulación (%)	92,0 ± 0,25
Parámetros de tamaño de glóbulos: *(lente 45 mm)*	
$d_{(v, 0,5)}$ (µm)	1,20 ± 0,02
$d_{(v, 0,9)}$ - $d_{(v, 0,1)}$ (µm)	33,45 ± 2,87
D [3,2] (µm)	0,76 ± 0,01
ASE (μm^{-1})	7,89 ± 0,10
Actividad de agua, a_w	0,16 ± 0,005
Temperatura de transición vítrea, Tg (ºC)	63,86 ± 0,49
Parámetros de densidad:	
A (Kg/m^3)	385 ± 3,1
P (Kg/m^3)	501 ± 7
Aire intersticial (mm^3/g)	599 ± 28
Compresibilidad (%)	23,08 ± 2,7
Solubilidad, t (s)	20 ± 0,6
pH	7,09 ± 0,05

Los resultados expresan la media ± la desviación estándar de la media de 3 muestras.

La evaluación de los lípidos totales extraídos no mostró diferencias en estabilidad oxidativa mientras que el análisis específico de la fracción superficial, que aunque minoritaria (8% de los lípidos totales) es más susceptible a la oxidación, mostró un aumento significativo de compuestos de oxidación a todas las temperaturas, y fue consistente con la aparición de rancidez en algunas muestras. La Figura 4 resume los resultados obtenidos tras el análisis de las fracciones superficiales. La pérdida de tocoferoles, como se observa en la Figura 5, fue consistente con el aumento de los niveles de oxidación observados. Estos resultados demuestran la necesidad de analizar separadamente la fracción lipídica superficial en fórmulas infantiles en polvo para determinar su estado de

oxidación. De hecho, los estudios previos realizados en fórmulas infantiles en polvo han mostrado a menudo resultados contradictorios debido a que sólo se analizó la grasa total extraída. Por ejemplo, detección de rancidez en muestras aparentemente no oxidadas, (Manglano y col., 2005; García-Llatas y col., 2006; Chávez-Serrín y col., 2008a).

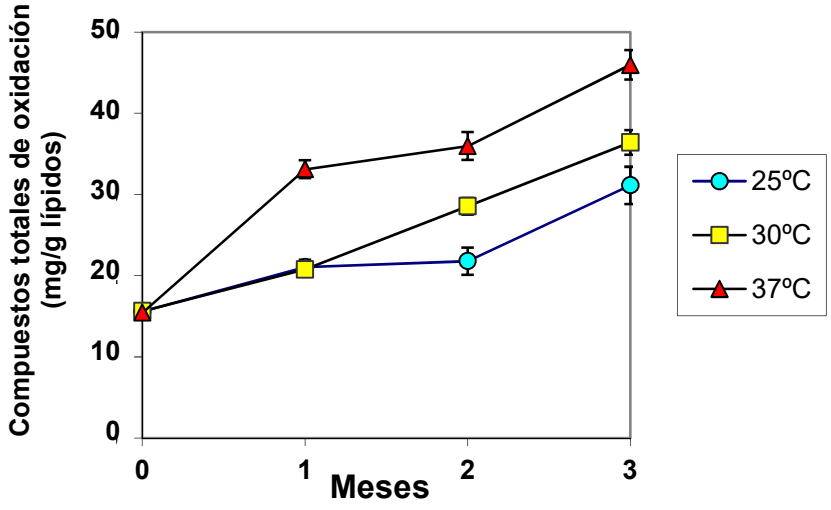

Fig. 4.- Evolución de compuestos totales de oxidación en la fracción lipídica superficial de fórmulas infantiles en polvo durante su almacenamiento a diferentes temperaturas.

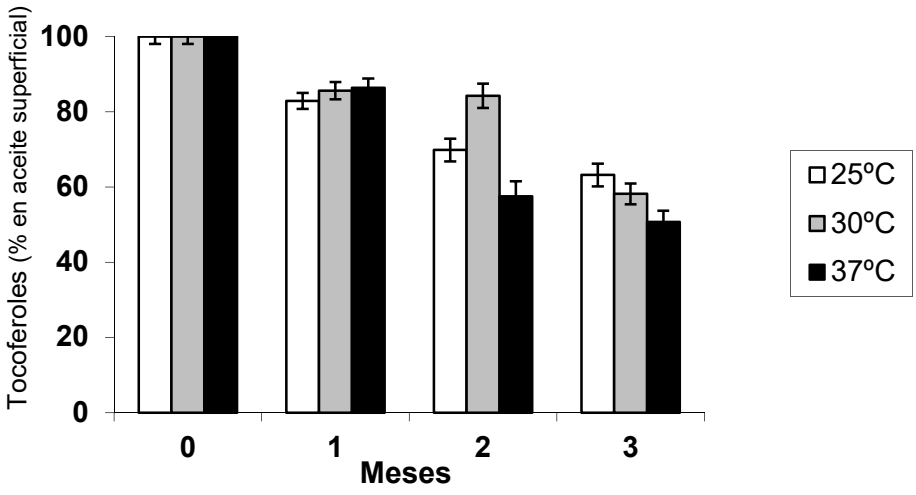

Fig. 5.- Evolución del contenido remanente de tocoferoles en la fracción lipídica superficial de fórmulas infantiles en polvo durante su almacenamiento a diferentes temperaturas.

La Tabla 4 recoge los resultados obtenidos tras almacenar 10 latas de fórmulas infantiles del mismo lote en sus envases originales a 25ºC durante 30 meses.

Tabla 4.- Evaluación de la oxidación de las fórmulas infantiles almacenadas a 25ºC durante 30 meses.

	Fracción libre			Fracción encapsulada		
Muestra	IP (meq O/kg)	POL (%)	TOC (mg/kg)	IP (meq O/kg)	POL (%)	TOC (mg/kg)
1	32,3	0,6	365	4,8	0,5	392
2	88,0	0,7	345	16,4	0,7	384
3	48,2	0,6	353	14,8	0,7	386
4	28,2	0,6	359	10,8	0,7	384
5	51,8	0,7	348	8,4	0,7	390
6	60,1	0,7	360	10,3	0,7	387
7	88,6	0,9	354	3,6	0,5	393
8	68,2	0,6	362	8,2	0,7	396
9	124	1,0	336	6,4	0,7	395
10	108	1,0	340	16,5	0,6	385
Media ± SEM	69,74 ± 10,06a	0,7 ± 0,05a	352 ± 3,08a	10,0 ± 1,47b	0,7 ± 0,03a	391 ± 2,15b

SEM: desviación estándar de la media (n=5).
Diferentes letras en una misma fila indican diferencias significativas ($p<0,05$).

Aunque en general los valores obtenidos difieren considerablemente entre las muestras, se encontraron diferencias significativas en los valores de índice de peróxidos entre las fracciones libre o superficial y encapsulada de las fórmulas. Estos resultados están de acuerdo con los obtenidos en condiciones aceleradas a 25, 30 y 37ºC en presencia de aire donde también se observó la mayor susceptibilidad a la oxidación de la fracción superficial. Sin embargo, estas muestras almacenadas en sus envases originales a 25ºC no habían alcanzado todavía el final del periodo de inducción a los 30 meses y en ninguna se detectó olor a rancio.

El efecto protector de la atmósfera inerte quedó claramente reflejado en que para la misma temperatura, 25ºC, las fracciones libres o superficiales de las muestras en bolsas habían perdido en 3 meses un 60% de sus tocoferoles iniciales (Figura 5) mientras que las mismas muestras en sus envases originales sólo perdieron un 8% aproximadamente en 30 meses (Tabla 4).

En resumen, el análisis de la fracción superficial del aceite en las fórmulas reveló que la rancidez, cuando es detectada, se debe a su oxidación preferencial. Además, aun cuando su nivel de oxidación sea bajo, su determinación, así como la de los antioxidantes remanentes, es esencial para determinar el periodo de vida útil de las fórmulas infantiles.

4.4.- Evaluación de la oxidación en el aceite rico en CLA adicionado a productos lácteos funcionales. Identificación y cuantificación de los compuestos de oxidación formados, volátiles y no volátiles, y estudios de cinética de oxidación.

Los resultados discutidos en este apartado forman parte de las siguientes publicaciones:

- García-Martínez MC, Márquez-Ruiz G, Fontecha J and Gordon MH. Volatile oxidation compounds in a conjugated linoleic acid-rich oil (2009) *Food Chemistry* 113: 926-931.
- Márquez-Ruiz G, Holgado F, Ruiz-Méndez MV, Velasco J and García-Martínez MC. Oxidation of a functional, CLA-rich oil: determination of volatile and non-volatile compounds (2016) *European Food Research and Technology* 242:1993-2000.

Los productos lácteos funcionales con ácido linoleico conjugado mostraron un comportamiento oxidativo muy diferente a lo esperado en los estudios recogidos en el apartado 4.1. Con objeto de profundizar en su estudio y contribuir a su debido control de calidad, se han realizado diferentes ensayos en muy distintas condiciones cuya discusión general se describe a continuación.

Se ha utilizado el aceite Tonalin TG80 (contenido del 80% en triacilgliceroles), suministrado por la empresa Cognis, ya que es el adicionado como aceite rico en CLA en los productos lácteos evaluados. El aceite Tonalin es obtenido mediante isomerización alcalina del aceite de cártamo, también utilizado en estos estudios con fines comparativos. El contenido de ácido linoleico (C18:2 9*c*, 12*c*) (74,7%) en el aceite de cártamo es similar al del contenido total de CLA (76,8%) (38,2% de C18:2 9*c*, 11*t* y 38,6% de C18:2 10*t*, 12*c*) en el aceite Tonalin.

Los ensayos de oxidación se realizaron a 100ºC en Rancimat, a 60ºC y 40ºC en estufa y a temperatura ambiente (25ºC). En todos los ensayos, la evolución de la formación de compuestos primarios y secundarios de oxidación mostró enormes diferencias entre el aceite Tonalin y su precursor aceite de cártamo, independientemente de las muy diferentes condiciones seleccionadas.

Como ejemplo de las cinéticas de oxidación encontradas, las Figuras 6 y 7 muestran la evolución de compuestos no volátiles primarios (índice de peróxidos) y secundarios (polímeros) (Figura 6), y la evolución de compuestos secundarios volátiles (pentanal, hexanal, heptanal, *trans* 2-heptenal, *trans* 2-octenal y *trans* 2-nonenal (Figura 8), junto con la pérdida de tocoferoles, en ambos aceites a 40ºC.

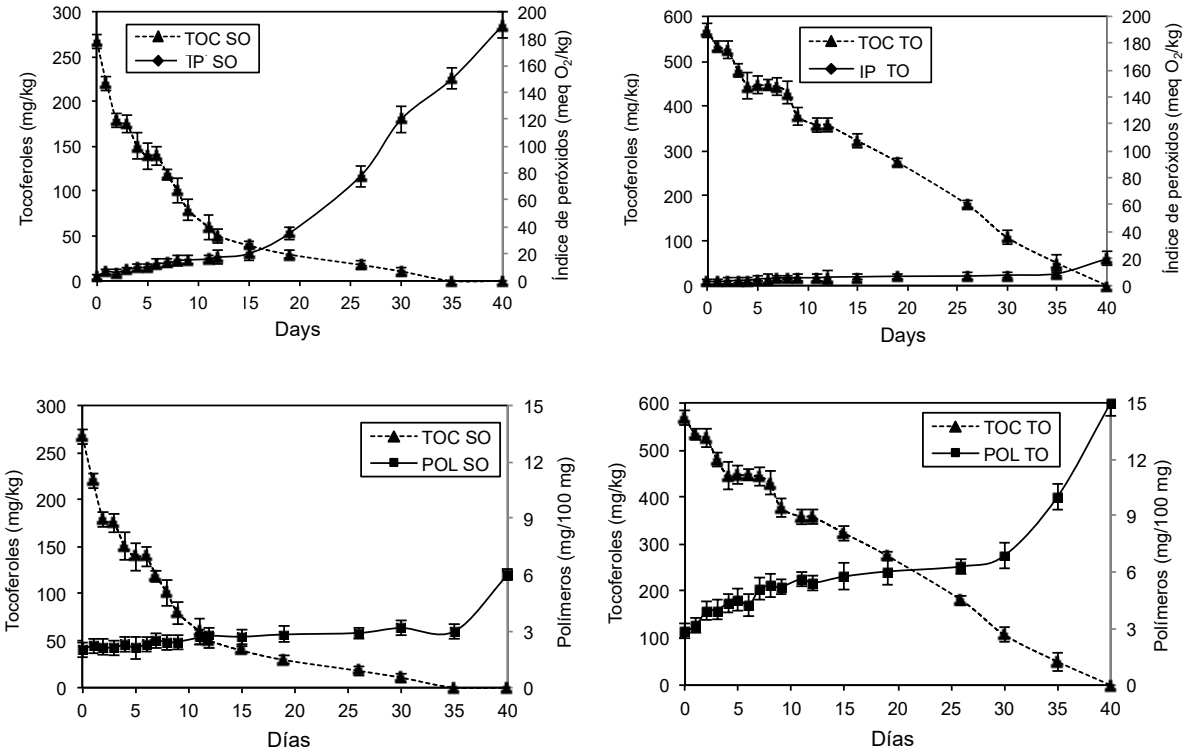

Fig. 6.- Evolución de compuestos de oxidación no volátiles (POL: polímeros, IP: índice de peróxidos) y tocoferoles (TOC) en aceites de cártamo (SO) y Tonalín (TO) a 40ºC.

El índice de peróxidos del aceite de cártamo mostró un aumento progresivo desde el principio y cuando se agotaron los tocoferoles, alcanzó un valor de 152 meq O_2/kg, mientras que en el aceite Tonalin el IP apenas aumentó durante el periodo de inducción y cuando se agotaron los tocoferoles, el IP sólo era 19 meq O_2/kg. En cuanto a los polímeros, ocurrió lo contrario, sus niveles permanecieron prácticamente constantes y por debajo del 3% durante el periodo de inducción en el aceite de cártamo y su aumento significativo marcó el inicio de la fase de oxidación avanzada, tal y como era de esperar, mientras que en el aceite Tonalin

los polímeros aumentaron desde el principio y alcanzaron niveles en torno al 15% en el punto de agotamiento de los tocoferoles.

La Figura 7 refleja las enormes diferencias encontradas entre los aceites al final del periodo de inducción, aproximadamente 35 días en ambos casos. Es interesante comentar además que los polímeros encontrados en el aceite Tonalin fueron casi exclusivamente trímeros y oligómeros de elevado peso molecular, que eluyen a tiempos de retención inferiores a los 12 min, y esto se observó desde el principio del curso de la oxidación.

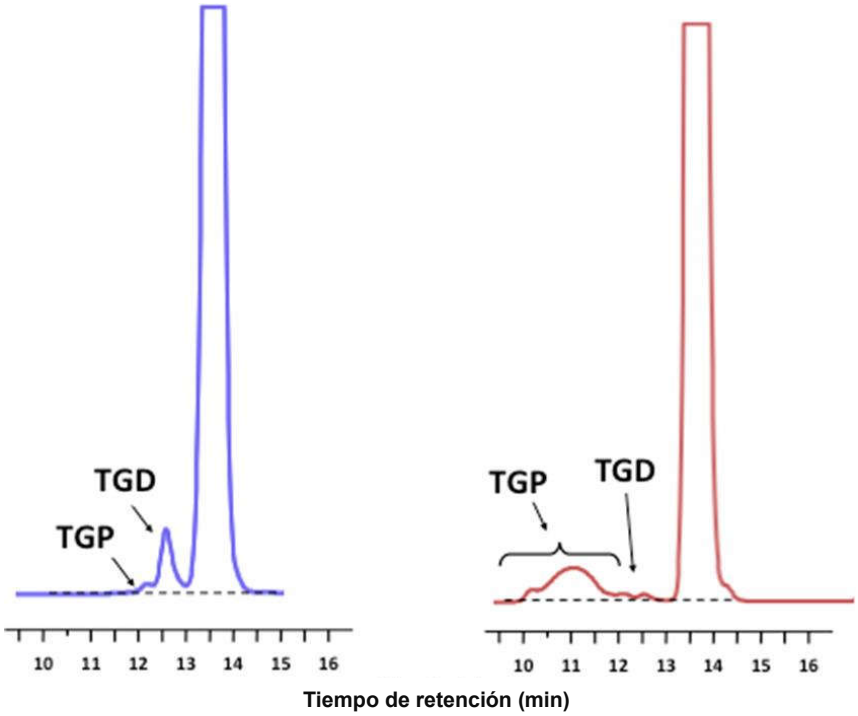

Fig. 7.- Perfil en cromatografía de exclusión molecular de dímeros (TGD) y polímeros (TGP) en aceite de cártamo (en azul) y aceite Tonalín (en rojo) tras 35 días a 40ºC.

Así mismo, se determinaron los compuestos de oxidación volátiles a distintas temperaturas y la Figura 8 muestra los resultados obtenidos a 40°C.

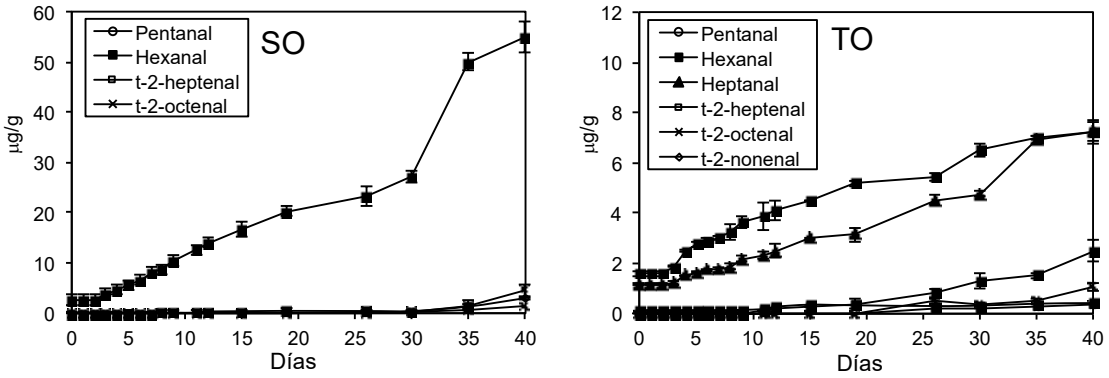

Fig. 8.- Evolución de compuestos de oxidación volátiles en aceites de cártamo (SO) y Tonalín (TO) a 40ºC.

En el aceite de cártamo, los compuestos encontrados fueron los esperados teóricamente de la escisión de los radicales alcoxilos derivados de los hidroperóxidos más abundantes, entre los cuales el hexanal es el mayoritario (Frankel, 2005). En cambio, en el aceite Tonalin el hexanal y el heptanal se formaron en cantidades similares y se formó también *t*-2-nonenal. La presencia de heptanal y *t*-2-nonenal no puede ser fácilmente justificada en base a la teoría de los hidroperóxidos porque sólo podría explicarse su formación a partir de hidroperóxidos de estructura improbable (Hamalainen y col., 2002). Sin embargo, podrían haberse formado por rutas alternativas, mediante la escisión de dioxoetanos formados a través de cicloadiciones 1,2 con oxígeno, tal y como se ha sugerido anteriormente (Yurawecz y col., 2003). Por otra parte, se formaron cantidades mucho más elevadas de volátiles en el aceite de cártamo que en Tonalin.

En la Figura 9 se muestra el inesperado perfil de volátiles obtenido en el aceite Tonalin. La detección de heptanal como marcador de oxidación de sustratos con ácido linoleico conjugado fue propuesta por primera vez a raíz de la publicación de estos resultados y ha sido ampliamente aceptada utilizada por otros investigadores (Giua y col., 2013; Yetella y col., 2012; Costa y col., 2015). Así mismo, la baja formación de volátiles y el muy distinto perfil encontrado en CLA podría explicar la imperceptible rancidez en los alimentos enriquecidos en CLA, hasta el momento erróneamente atribuida a su bajo nivel de oxidación.

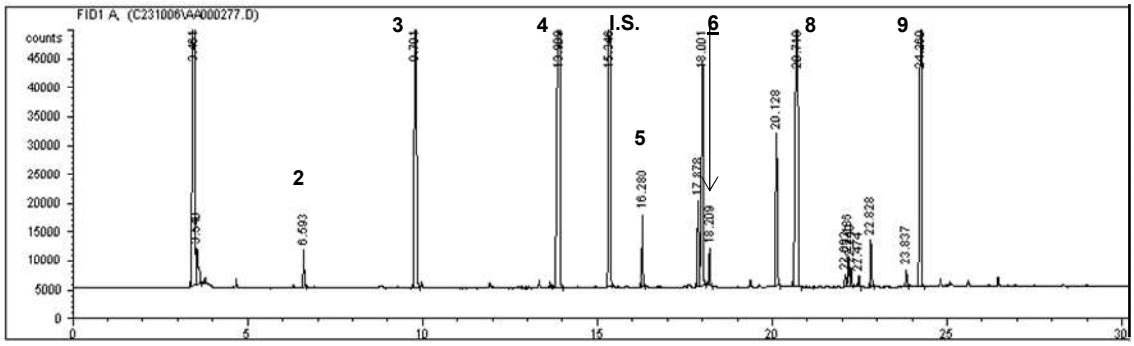

Fig. 9.- Perfil en cromatografía de gases con microextracción en fase sólida de los compuestos de oxidación volátiles del aceite Tonalin oxidado a 40ºC durante 10 días. I.S.: estándar interno, 1, hexano; 2, pentanal; 3, hexanal; 4, heptanal; 5, *t*-2-heptenal; 6, octanal; 7, limoneno; 8, *t*-2-octenal; 9, *t*-2-nonenal.

Los resultados obtenidos en las condiciones aceleradas del Rancimat (Figura 10), permitieron obtener similares conclusiones.

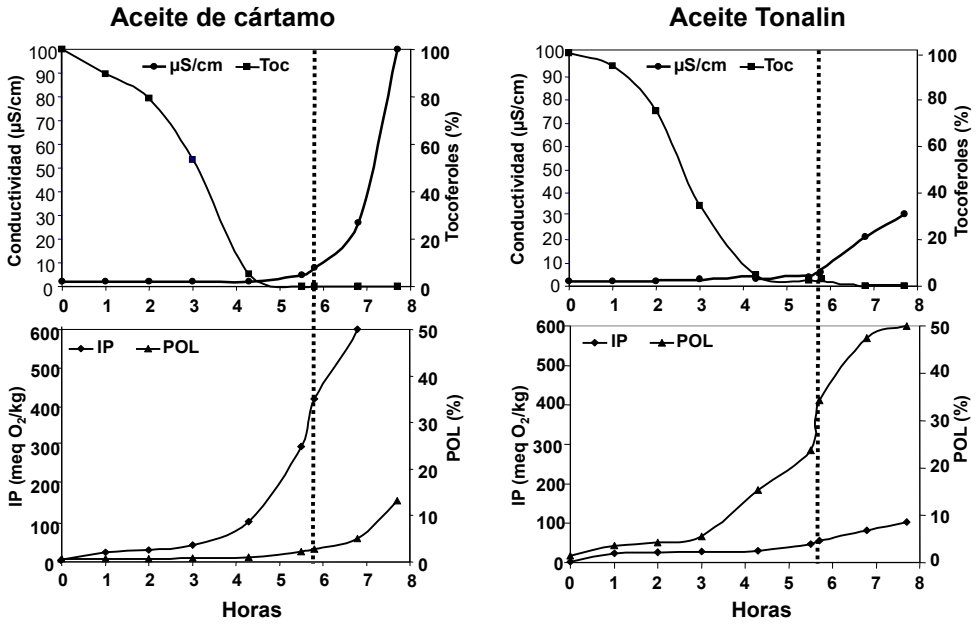

Fig.10.- Evolución de la conductividad, pérdida de tocoferoles, índice de peróxidos y polímeros en Rancimat a 100°C y flujo de aire de 20 L/hora.

En Rancimat, el final del periodo de inducción, proporcionado automáticamente por el equipo y basado en el aumento brusco de la conductividad debida al inicio de la formación de ácidos orgánicos volátiles, fue similar en ambos aceites (prácticamente 6 horas) pero en ese punto el aceite de cártamo tenía 421 meq O_2/kg de índice de peróxidos frente a 38 meq O_2/kg en el aceite Tonalin y éste ya

contenía 32.9% de polímeros frente a sólo 2.1% en el caso del aceite de cártamo.

Los resultados obtenidos en referencia a la oxidación del CLA han permitido proponer mecanismos de oxidación alternativos para estos sustratos de enlaces conjugados, como se muestra en el esquema incluido en la Figura 11.

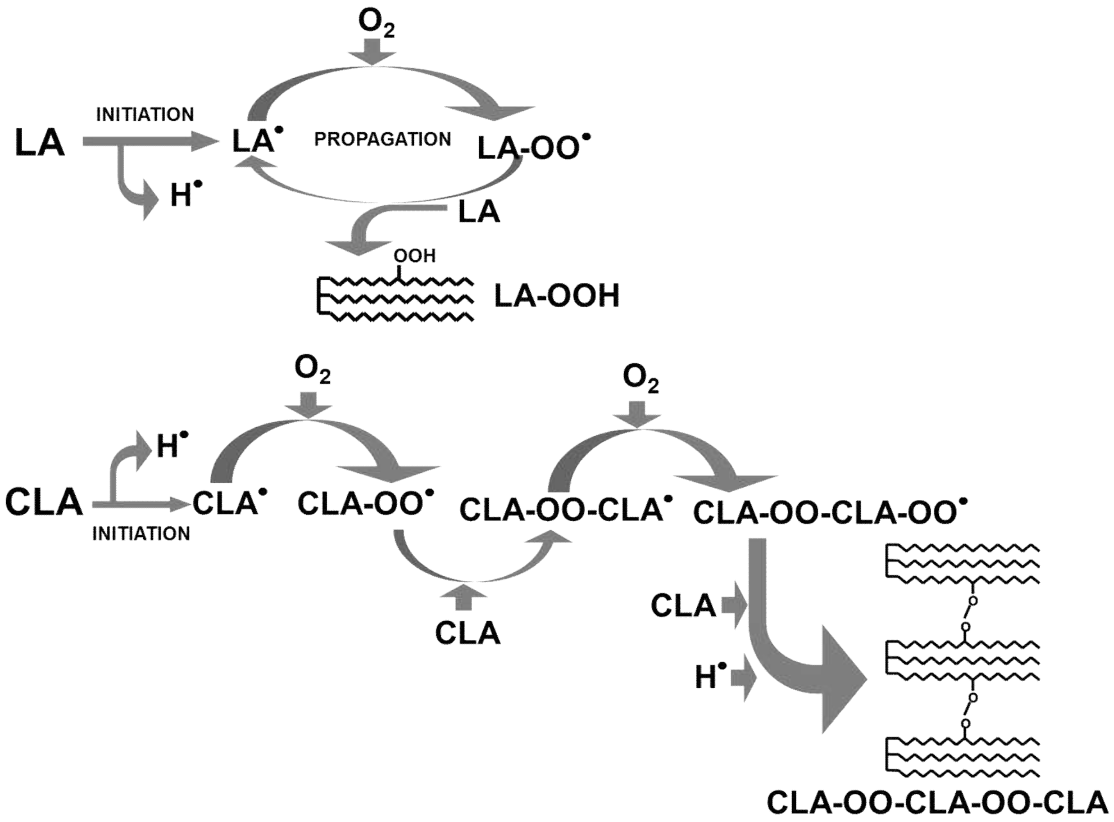

Fig. 11.- Representación esquemática de la formación de hidroperóxidos (LA-OOH) a partir del ácido linoleico (LA) y mecanismo propuesto para la formación de hidroperóxidos poliméricos (CLA-OO-CLA-OO-CLA) a partir del CLA.

Las diferencias encontradas pueden ser explicadas en términos de energía de disociación de enlaces (Oyman y col., 2005). Para el ácido linoleico (LA) la reacción más favorable del radical peroxilo (LA-OO•) formado en la etapa de propagación es la abstracción del hidrógeno del grupo alílico (272 kJ/mol) para formar hidroperóxidos (LA-OOH) como productos de oxidación primaria. Sin embargo, en el ácido linoleico conjugado (CLA) el mecanismo de reacción más favorable del radical peroxilo (CLA-OO•) es la adición al doble enlace conjugado (284 kJ/mol) ya que en este caso la abstracción del hidrógeno del grupo alílico

requeriría más energía (322 kJ/mol). Además, la adición del radical peroxilo al sistema dieno conjugado conduce a la formación de radicales alílicos estabilizados por resonancia (CLA-OO-CLA•). Estas diferencias en mecanismos de oxidación justificarían las bajas cantidades de hidroperóxidos encontrados en el aceite Tonalin a favor de la formación de peróxidos poliméricos (CLA-OO-CLA-OO-CLA), la ruta predominante. Estos polímeros no pueden ser por tanto considerados productos de terminación característicos de estados de oxidación avanzada sino compuestos primarios formados durante la etapa de propagación. Por otra parte, el mecanismo propuesto también justificaría los bajos niveles de volátiles encontrados en el aceite Tonalin en contraste con la acumulación de hidroperóxidos precursores en el aceite de cártamo (Figura 8).

En resumen, los resultados obtenidos demuestran que existen mecanismos de oxidación diferentes entre los aceites ricos en ácido linoleico, que se oxidan según la teoría ampliamente aceptada a través de la formación de hidroperóxidos, y los aceites ricos en ácido linoleico conjugado, en los cuales los compuestos primarios de oxidación no son hidroperóxidos sino polímeros con enlaces peróxido de elevado peso molecular. Estos resultados, además de contribuir de manera importante al conocimiento de la cinética de oxidación de estos sustratos, demuestran la invalidez del índice de peróxidos como medida de control de oxidación de aceites ricos en CLA, la única que consta en las especificaciones de los productos comercializados.

4.5.- Desarrollo de estrategias para aumentar la estabilidad oxidativa del CLA, mediante la adición de antioxidantes fenólicos, y mediante microencapsulación en matriz láctea.

Los resultados discutidos en este apartado forman parte de las siguientes publicaciones:

- Márquez-Ruiz G, García-Martínez MC, Holgado F and Velasco J. Effectiveness of α-, γ - and δ-tocopherol in a CLA-rich oil (2014) *Antioxidants* 3:176-188.

- Holgado F, García-Martínez MC, Velasco J, Ruiz-Méndez MV and Márquez-Ruiz G. Microencapsulation of conjugated linoleic acid (CLA)-rich oils with skimmed milk components protects against polymerization. *Journal of the American Oil Chemists Society.* DOI:10.1002/aocs.12146

Con objeto de aumentar la estabilidad oxidativa de los aceites ricos en CLA se han utilizado dos estrategias, la adición de diferentes tipos de tocoferoles y sinergistas, y la técnica de microencapsulación.

4.5.1.- Adición de antioxidantes a aceites ricos en ácido linoleico conjugado.

Los antioxidantes seleccionados fueron el alfa, delta y gamma-tocoferol, así como sus combinaciones con la mezcla sinérgica APL (palmitato de ascorbilo + lecitina), que se adicionaron a los aceites una vez desprovistos de sus antioxidantes, mediante cromatografía en columna de alúmina. El único precedente encontrado en este contexto es un trabajo en el cual se añadió alfa-tocoferol a aceites ricos en CLA, encontrándose un aumento de la estabilidad pero resultados contradictorios entre las determinaciones utilizadas (Tsuzuki y col., 2004).

Los ensayos se realizaron en Rancimat, de demostrada utilidad como método de oxidación acelerada ya que proporciona resultados comparativos entre los aceites similares a los obtenidos a temperaturas inferiores o ambiente. La Figura

12 muestra la eficacia de las distintas mezclas de antioxidantes en el aceite Tonalin y en el aceite de cártamo.

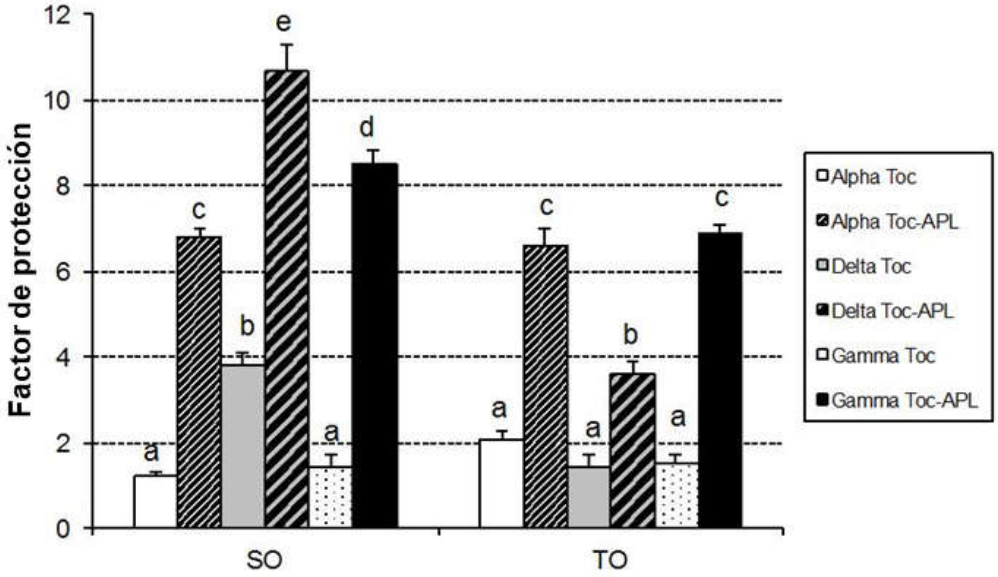

Fig. 12.- Factores de protección (Rancimat) obtenidos en aceites de cártamo (SO) y Tonalin (TO) desprovistos de antioxidantes y adicionados con homólogos alfa, delta y gamma de tocoferol y la mezcla sinérgica palmitato de ascorbilo-lecitina (APL). Las letras diferentes indican diferencias significativas entre las muestras preparadas con el mismo aceite.

Como puede observarse, la protección de la mayoría de los sistemas antioxidantes fue inferior para el aceite Tonalin y especialmente sorprendente fue encontrar que el delta-tocoferol mostró la misma y baja actividad que los demás tipos de tocoferol, al contrario de lo encontrado en el aceite de cártamo y en general ampliamente demostrado en una gran variedad de aceites y grasas. Así mismo, la adición de APL fue en general menos protectora en el aceite Tonalin.

Los resultados demuestran que el delta-tocoferol, muy efectivo en aceites poliinsaturados, lo es mucho menos en el aceite Tonalin. Así mismo, la combinación de delta-tocoferol y APL, utilizada normalmente en el aceite Tonalin adicionado a productos lácteos funcionales, ejerce una protección marcadamente inferior a la esperada, que en cambio sí es efectiva en el aceite de cártamo. Es por tanto esencial investigar la actividad antioxidante o antipolimerizante de antioxidantes alternativos que sean eficaces en aceites ricos en ácido linoleico conjugado.

4.5.2.- Microencapsulación de aceites ricos en ácido linoleico conjugado con matriz láctea.

Los ensayos con aceite de Tonalin microencapsulado (TM), suministrados por una empresa, se realizaron aplicando la separación diferencial de fracción libre y encapsulada. Las características físico-químicas de las muestras de los dos lotes suministrados (TM1 y TM2) fueron evaluadas y se recogen en la Tabla 5. En el caso de las muestras TM2, la Figura 13 ilustra la distribución de tamaños de glóbulo de aceite obtenida y la curva de calorimetría diferencial que denota la temperatura de transición vítrea.

El contenido de aceite en ambos lotes de aceite de Tonalin microencapsulado fue similar, alrededor del 8%. Las únicas características significativamente diferentes entre ambos lotes fueron la eficacia de encapsulación y la temperatura de transición vítrea, ambas superiores en el lote TM2.

Tabla 5.- Características físicoquímicas del aceite de Tonalin microencapsulado ™

	TM 1	**TM 2**
Distribución lipídica:		
Aceite Total (g/100 g	8,12 ± 0,50	8,15 ± 0,45
Aceite Libre (g/100 g	0,97 ± 0,03 **a**	0,51 ± 0,02 **b**
Eficacia de Encapsulación (%)	88,0 ± 1,62 **a**	93,7 ± 1,30 **b**
Parámetros de tamaños de glóbulos: *(lente 45 mm)*		
$d_{(v, 0,5)}$ (µm)	0,62 ± 0,01 **a**	0,74 ± 0,02 **b**
$d_{(v, 0,9)}$ - $d_{(v, 0,1)}$ (µm)	10,52 ± 1,26	9,31 ± 0,87
D [3,2] (µm)	0,60 ± 0,04	0,58 ± 0,05
ASE (μm^{-1})	10,00 ± 0,14	10,34 ± 0,30
Actividad de agua, a_w	0,20 ± 0,003	0,18 ± 0,002
Temperatura de transición vítrea, Tg (ºC)	63,87 ± 0,46 **a**	75,38 ± 0,62 **b**
Parámetros de color:		
L*(D65)	94,06 ± 0,14	94,39 ± 0,11
a*(D65)	-1,73 ± 0,04	-1,72 ± 0,04
b*(D65)	11,99 ± 0,14 **a**	13,32 ± 0,15 **b**
Solubilidad, t (s)	260 ± 0,7 **a**	230 ± 0,6 **b**
pH	6,7 ± 0,04	6,7 ± 0,05

Los resultados expresan la media ± desviación estándar de la media de 3 muestras.Letras distintas en una misma fila indican diferencias significativas ($p<0,05$).

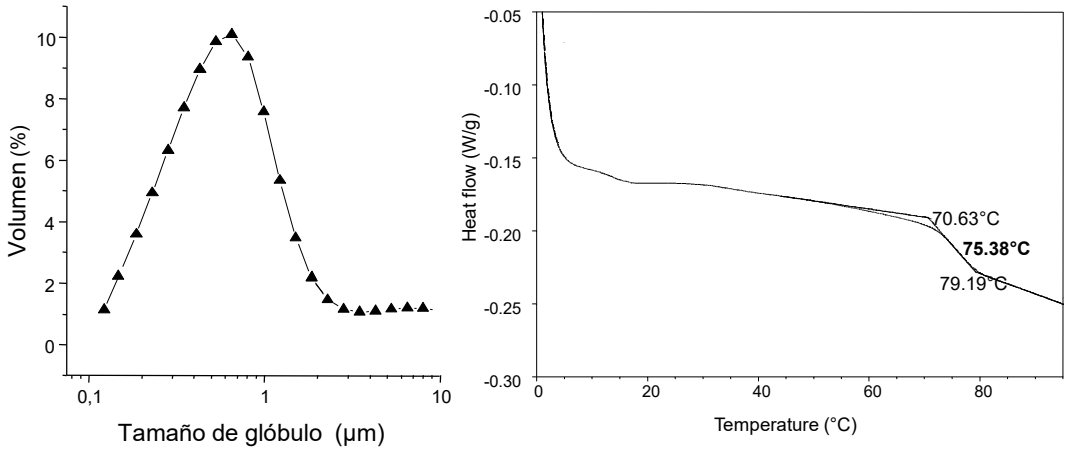

Fig. 13.- Distribución de tamaños de glóbulos de aceite en la emulsión reconstituida en agua (A) y curva DSC (B) de la muestra TM 2.

Las muestras de aceite de Tonalin microencapsulado fueron almacenadas a 30°C, en oscuridad y a sequedad. El comportamiento oxidativo de las fracciones libre y encapsulada de TM1 y TM2 fue similar en cuanto a que la fracción libre se oxidó mucho más rápidamente en ambos casos. Como ejemplo, la Figura 14 muestra la evolución de compuestos de polimerización y tocoferoles en las fracciones libre y encapsulada de la muestra TM2.

En las fracciones de aceite libre o superficial la polimerización comenzó en los primeros días, en cambio en las de aceite encapsulado no ocurrió hasta los 50 días. Este hecho pone de relieve la protección ejercida por la matriz sobre la fracción de aceite encapsulado frente a la oxidación. Se observó, además, que los niveles alcanzados de dímeros y polímeros cuando se agotaron los antioxidantes fueron superiores en la fracción encapsulada (8%) que en la fracción de aceite libre (4%).Todos estos resultados son novedosos ya que, además de que se han publicado muy escasos trabajos sobre microencapsulación del CLA, en éstos sólo se ha utilizado el CLA en su forma ácida y no aceites rico en CLA, y nunca se ha realizado la separación de las fracciones superficial y encapsulada ni se ha utilizado la detección de polímeros como marcador de la oxidación (Kim y col., 2000; Jiménez y col., 2004; 2006; Lee y col., 2009; Costa y col., 2015).

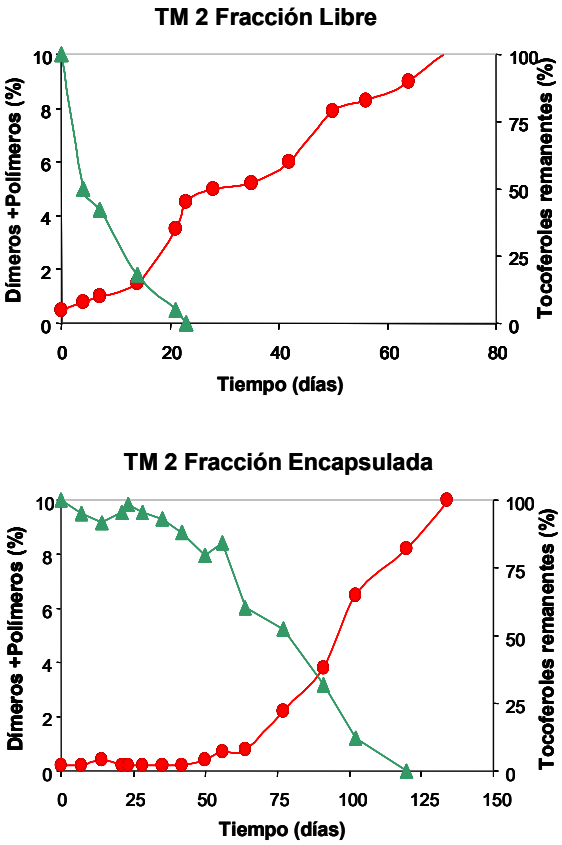

Fig. 14.- Evolución de compuestos poliméricos y tocoferoles en la fracción libre y encapsulada de Tonalín microencapsulado, a 30 ºC. Dímeros+Polímeros (●) y Tocoferoles (▲).

Las diferencias en la relación entre tocoferoles remanentes y las cantidades de dímeros y polímeros entre las fracciones de aceite libre y encapsulado se observaron en todo el curso de oxidación, y están representadas en la Figura 15 para ambos lotes de muestras.

Estos resultados se atribuyen al carácter discontinuo de la oxidación en la fracción del aceite encapsulado, es decir, a la coexistencia de gotículas de aceite encapsulado con diferentes estadíos de oxidación (desde gotículas de aceite poco oxidadas con cantidades elevadas de tocoferoles hasta gotículas de aceite muy oxidadas, ya desprovistas de tocoferoles y con cantidades significativas de polímeros).

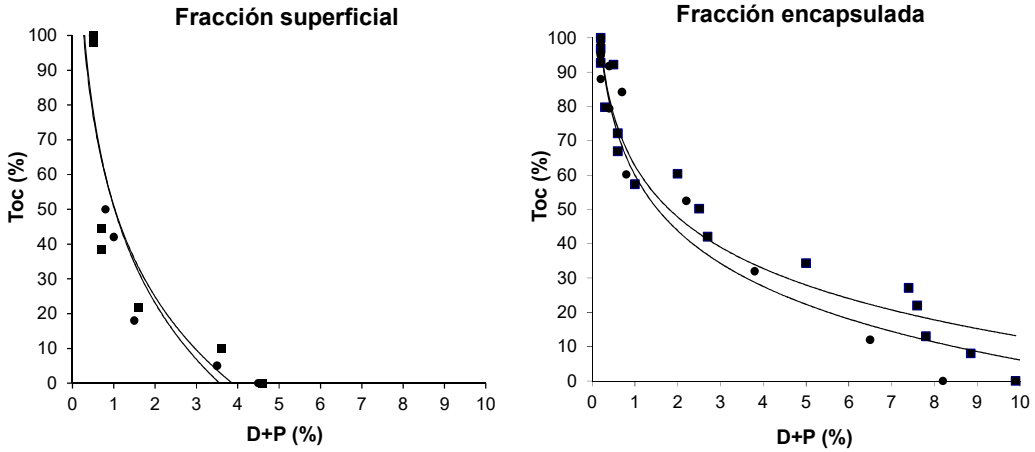

Fig. 15.- Evolución de la relación entre tocoferoles remanentes y cantidades de compuestos de polimerización en las fracciones de aceite libre y encapsulado extraídas de muestras TM 1 (cuadrados) y TM 2 (círculos) sometidas a 30 ºC, HR de 0 % y oscuridad.

Los resultados demuestran que la microencapsulación es una estrategia tecnológica muy eficaz para proteger aceites ricos en CLA utilizados como ingredientes funcionales en productos lácteos, ya que la estabilidad oxidativa de la fracción de aceite libre es mucho menor. Además, puede concluirse que es esencial obtener elevadas eficacias de encapsulación debido a que la oxidación de la fracción de aceite libre, aun siendo la minoritaria, determina la vida útil del producto.

5.- Conclusiones.

Los resultados obtenidos en este trabajo han permitido extraer las siguientes conclusiones:

1.- La metodología basada en una combinación de técnicas cromatográficas, aplicada a productos lácteos por primera vez, ha permitido evidenciar la elevada susceptibilidad a la oxidación lipídica de los productos lácteos enriquecidos en ácidos grasos poliinsaturados omega-3 y ácido linoleico conjugado. Así mismo, se ha constatado que en los productos lácteos convencionales analizados al final de su vida útil no se observó ningún cambio oxidativo.

2.- En las fórmulas infantiles, la adición de ácidos grasos poliinsaturados omega-3 disminuyó considerablemente su estabilidad y que el principal agente protector de su formulación fue la fracción proteica, especialmente el caseinato sódico.

3.- El tratamiento de esterilización no indujo cambios oxidativos en fórmulas infantiles de inicio y continuación elaboradas con composición similar a las comercializadas. No obstante, se observaron disminuciones significativas de tocoferoles en las fórmulas de inicio con la consecuente pérdida de protección frente al posible deterioro oxidativo durante el posterior periodo de comercialización.

4.- El análisis de la fracción superficial del aceite en las fórmulas infantiles reveló que la rancidez, cuando fue detectada, se debió a su oxidación preferencial. De ahí que el estado de oxidación de la fracción superficial, aun siendo ésta minoritaria, determine la vida útil de las fórmulas infantiles.

5.- Los ensayos de oxidación realizados en condiciones aceleradas a 100ºC y con flujo de aire, a 60ºC y 40ºC en estufa y a temperatura ambiente (25ºC) han demostrado que la evolución de la formación de compuestos primarios y secundarios de oxidación es muy diferente en aceites ricos en ácido linoleico

conjugado respecto a su precursor aceite de cártamo, rico en ácido linoleico convencional, no conjugado.

6.- Los resultados obtenidos en referencia a la oxidación del ácido linoleico conjugado han permitido proponer mecanismos de oxidación alternativos para estos sustratos de enlaces conjugados, que no se oxidan preferentemente a través de la formación de hidroperóxidos, sino mediante la formación de polímeros con enlaces peróxido de elevado peso molecular ya desde el inicio del proceso oxidativo.

7.- La ineficacia del índice de peróxidos como medida de control de oxidación de aceites ricos en ácido linoleico conjugado ha quedado demostrada y justificada por la ruta alternativa de oxidación propuesta. Por tanto, se propone sustituir la medida del índice de peróxidos, la única que consta en las especificaciones de los productos comercializados, por determinaciones indicativas del grado de polimerización.

8.- La detección del compuesto volátil heptanal fue propuesta como marcador de oxidación del ácido linoleico conjugado por primera vez en este trabajo y, tras su publicación, su utilidad ha sido constatada por otros investigadores.

9.- La baja formación de volátiles y el muy distinto perfil encontrado en ácido linoleico conjugado han permitido dar explicación a la imperceptible rancidez de los alimentos enriquecidos en ácido linoleico conjugado.

10.- La eficacia de los antioxidantes delta-tocoferol y su combinación con palmitato de ascorbilo y lecitina ha demostrado ser muy inferior a lo esperado, en contraste con la elevada protección conferida a otros aceites poliinsaturados. Dado que éstos son los antioxidantes normalmente utilizados en los aceites ricos en ácido linoleico conjugado adicionados a productos lácteos funcionales, los resultados obtenidos demuestran la necesidad de investigar la actividad antioxidante o antipolimerizante de antioxidantes alternativos.

11.- Los resultados demuestran que la microencapsulación con matriz láctea es una estrategia tecnológica muy eficaz para proteger aceites ricos en ácido linoleico conjugado utilizados como ingredientes funcionales en productos lácteos.

12.- En los aceites ricos en ácido linoleico conjugado microencapsulados, las fracciones libre y encapsulada mostraron un comportamiento oxidativo muy diferente. En la fracción libre, la oxidación tiene lugar de manera continua, con un periodo de inducción definido. Sin embargo, la fracción de aceite encapsulado muestra una oxidación discontinua ya que se encuentran cantidades elevadas de compuestos de polimerización existiendo aún niveles altos de antioxidantes. Estos resultados indican la coexistencia de gotículas de aceite que se oxidan a velocidades de reacción muy diferentes.

En resumen, la contribución general de esta Tesis ha sido aumentar el conocimiento de los mecanismos de oxidación lipídica en productos lácteos, concretamente en los altamente susceptibles a esta alteración, es decir, los productos lácteos funcionales enriquecidos en ácidos grasos poliinsaturados omega-3 y ácido linoleico conjugado. Así mismo, se han propuesto nuevos métodos para evaluar el estado de oxidación de estos productos, cuyo control es esencial para garantizar que los lípidos bioactivos adicionados proporcionen los beneficios nutricionales esperados.

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